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Bending the Rules of Cell Protrusions

**Molecular Mechanisms and Biological Roles of Inverse-BAR Proteins in Cell Morphogenesis**

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Dissertationes bioscientiarum molecularium Universitatis Helsingiensis in Viikki
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ABBREVIATIONS

ABBA  actin binding protein with BAIP homology
ADF   actin depolymerizing factor
ADP   adenosine diphosphate
ALP   alkaline phosphatase
Arp   actin related protein
ATP   adenosine triphosphate
BAR   Bin-Amphiphysin-Rsv
Cc    critical concentration
Cdc42  Cell division control protein 42 homolog
CNS   central nervous system
CRIB  Cdc42/Rac interactive binding
D     dimension
DNA   deoxyribonucleic acid
DPH   1,6-diphenyl 1,3,5-hexatriene
DXA   dual-energy X-ray absorptiometry
E     embryonic day
EHEC  enteropathogenic E. coli
EM    electron microscopy
EMT   epithelial to mesenchymal transition
Ena/VASP enabled/vasodilator-stimulated phosphoprotein
ERM   ezrin/radixin/moesin
EHEC  enterohemorrhagic E. coli
F-actin filamentous actin
F-BAR  FCH BAR
FCH   FER/CIP4 homology
FRAP  fluorescence recovery after photobleaching
G-actin monomeric (globular) actin
β-GAL  β- galactosidase
GAP   GTPase activating protein
GDP   guanosine diphosphate
GEF   GDP/GTP exchange factor
GFP   green fluorescent protein
GST   glutathione S-transferase
GTP   guanosine triphosphate
GTPase guanosine triphosphatase
GUV   giant unilamellar vesicle
I-BAR  inverse BAR domain
IF    intermediate filament
IMD   IRSp53/MIM homology (IM) domain
IRSp53 insulin receptor tyrosine kinase substrate p53
IRTKS insulin receptor tyrosine kinase substrate
Kank  kidney ankyrin repeat-containing protein
Kd    dissociation constant
kDa   kilodalton
MDCK  Madin-Darby Canine Kidney Cells
MIM   missing-in-metastasis
MLV   multilamellar vesicle
mRNA  messenger RNA
MW    molecular weight
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NBD</td>
<td>7-chloro-4-nitrobenz-2-oxa-1,3-diazole</td>
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<tr>
<td>NPF</td>
<td>nucleation promoting factor</td>
</tr>
<tr>
<td>N-WASP</td>
<td>neural WASP</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAK</td>
<td>P21/Cdc42/Rac1-activated kinase</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDZ</td>
<td>post synaptic density</td>
</tr>
<tr>
<td>Pi</td>
<td>pyrophosphate, inorganic phosphate</td>
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<tr>
<td>PI</td>
<td>phosphoinositide</td>
</tr>
<tr>
<td>PIPK</td>
<td>phosphoinositide kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidyl inositol phosphate</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>Ptc</td>
<td>patched</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
</tr>
<tr>
<td>Rif</td>
<td>Rho in filopodia</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil forming kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC Homology 3 Domain</td>
</tr>
<tr>
<td>SiRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>VCA</td>
<td>Verprolin, Central, Acidic</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family Verprolin homologous</td>
</tr>
<tr>
<td>WH2</td>
<td>WASP homology domain 2</td>
</tr>
<tr>
<td>WIP</td>
<td>WASP interacting protein</td>
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LIST OF ORIGINAL PUBLICATIONS


* Equal contribution

The publications are referred to in the text by their roman numerals.
ABSTRACT

Plasma membrane adopts myriad of different shapes to carry out essential cellular processes such as nutrient uptake, immunological defence mechanisms and cell migration. Therefore, the details how different plasma membrane structures are made and remodelled are of the utmost importance. Bending of plasma membrane into different shapes requires substantial amount of force, which can be provided by the actin cytoskeleton, however, the molecules that regulate the interplay between the actin cytoskeleton and plasma membrane have remained elusive. Recent findings have placed new types of effectors at sites of plasma membrane remodelling, including BAR proteins, which can directly bind and deform plasma membrane into different shapes. In addition to their membrane-bending abilities, BAR proteins also harbour protein domains that intimately link them to the actin cytoskeleton. The ancient BAR domain fold has evolved into at least three structurally and functionally different sub-groups: the BAR, F-BAR and I-BAR domains.

This thesis work describes the discovery and functional characterization of the Inverse-BAR domains (I-BARs). Using synthetic model membranes, we have shown that I-BAR domains bind and deform membranes into tubular structures through a binding-surface composed of positively charged amino acids. Importantly, the membrane-binging surface of I-BAR domains displays an inverse geometry to that of the BAR and F-BAR domains, and these structural differences explain why I-BAR domains induce cell protrusions whereas BAR and most F-BAR domains induce cell invaginations. In addition, our results indicate that the binding of I-BAR domains to membranes can alter the spatial organization of phosphoinositides within membranes. Intriguingly, we also found that some I-BAR domains can insert helical motifs into the membrane bilayer, which has important consequences for their membrane binding/bending functions.

In mammals there are five I-BAR domain containing proteins. Cell biological studies on ABBA revealed that it is highly expressed in radial glial cells during the development of the central nervous system and plays an important role in the extension process of radial glia-like C6R cells by regulating lamellipodial dynamics through its I-BAR domain. To reveal the role of these proteins in the context of animals, we analyzed MIM knockout mice and found that MIM is required for proper renal functions in adult mice. MIM deficient mice displayed a severe urine concentration defect due to defective intercellular junctions of the kidney epithelia. Consistently, MIM localized to adherens junctions in cultured kidney epithelial cells, where it promoted actin assembly through its I-BAR and WH2 domains.

In summary, this thesis describes the mechanism how I-BAR proteins deform membranes and provides information about the biological role of these proteins, which to our knowledge are the first proteins that have been shown to directly deform plasma membrane to make cell protrusions.
### REVIEW TO THE LITERATURE

#### 1.1. Plasma membrane

Plasma membrane is mainly composed of lipids and proteins. It acts both as a physical barrier as well as an exchange platform between the cell and its surroundings. The characteristic nature of membrane is formed by the physical nature of amphipathic lipid molecules, which contain hydrophobic tails and hydrophilic headgroups. When lipids are exposed to aqueous environment, they spontaneously form bilayers by exposing their hydrophilic headgroups to the aqueous phase and forming a hydrophobic core from their tails.

In the plasma membrane, the outer leaflet facing the extracellular space is called the exoplasmic face and the inner leaflet is called the cytosolic face. There are hundreds of different lipids found in the plasma membrane that are defined by differences in the head group and/or in the length or degree of saturation of the acyl chain. The most common group of lipids found in the plasma membrane are phospholipids. Some phospholipids, like phosphatidylethanolamine (PE), have no net charge whereas phosphatidylserine, for example, has a negative net charge (Lodish et al., 1999). One of the corner stones of membrane biology was the introduction of the fluid mosaic model, which described the plasma membrane as a sea of lipids in which proteins are embedded, and which does not contain any long range order (Singer and Nicolson, 1972). More recently however, it has been proposed that certain lipids such as cholesterol and sphingomyelin are capable of forming clusters in plasma membrane called membrane rafts, which can act as platforms for various different events including membrane trafficking (Simons and Ikonen, 1997). Therefore, the current view describes plasma membrane as asymmetrical both in its lipid and protein distribution (Edidin, 2003).

Thermal motion permits lipid diffusion laterally and along its long axis, however migration from one leaflet to another is energetically highly unfavorable event and does not occur without the aid of proteins called flippases. The lateral diffusion is constricted by membrane spanning and membrane-bound proteins that are linked, for example, to the cytoskeleton (Lodish et al., 1999).

Importantly, lipid asymmetry between outer and inner leaflets can result in generation of membrane curvature due to specific characteristics of different lipid species (Kozlov, 2010). In addition, proteins can influence plasma membrane curvature in several different ways. The so called scaffolding mechanism describes how rigid membrane-binding proteins such as BAR domain proteins, which display intrinsic curvature, can bend the membrane to fit their intrinsic shape. The local spontaneous curvature mechanism describes an event where a given protein like epsin, for example, inserts an amphipathic motif into the bilayer, which can act as wedge that bends membrane due to local bilayer asymmetry. Also, integral membrane proteins, such as transmembrane receptors and channels can influence membrane curvature depending on their structural features. Moreover, cytoskeletal elements, such as actin or septin filaments can generate forces that bend the membrane. In addition, assemblies of polymerized coat proteins, such as clathrin are capable of stabilizing existing curvature (Kozlov, 2010; Tanaka-Takiguchi et al. 2009; Shibata et al. 2009; Doherty and McMahon 2008; Zimmerberg and Kozlov, 2006; McMahon and Gallop, 2005). Plasma membrane curvature can be described as ‘positive’ to refer membrane regions that fold inwards towards the cytoplasm or as ‘negative’ to
describe plasma membrane regions that bend outwards, away from the cytoplasm (Gallop and McMahon 2005 (Figure 1).

1.2. Phosphoinositides act as sub-cellular signposts

Phosphoinositides constitute a divergent class of phospholipids that has multiple roles in regulating a vast number of cellular events, ranging from membrane trafficking to apoptosis and the organization of the cytoskeleton (Bittar, 2006; Di Paolo and De Camilli, 2006; Niggli, 2005). Consequently, these lipids have a pronounced role in human diseases (Wymann and Schneiter, 2008). Phosphatidylinositol is composed of a d-myo-inositol-1-phosphate, which is linked to diacylglycerol. The inositol ring can be reversibly phosphorylated at positions D-3, D-4, or D-5 by phosphoinositide kinases and dephosphorylated by phosphoinositide phosphatases. In mammals, there are at least 19 different phosphoinositide kinases and 28 phosphoinositide phosphatases, which have overlapping tissue distributions and can give rise to seven distinct phosphoinositide species (PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂, and PI(3,4,5)P₃) (Sasaki et al., 2009). Distinct phosphoinositides are found at variable concentrations in specific sub-cellular membrane compartments. They can specify these membrane compartments by recruiting proteins that favour binding to specific phosphoinoside species. These protein domains that can specifically interact with certain phosphoinoside species include PH and PX-domains, which can be found in various proteins (Lemmon, 2008). Many of them, such as BAR domain containing protein sortin nexin 9 (SNX9), are involved in membrane and cytoskeleton remodelling (Pylypenko et al. 2007).

The most abundant phosphorylated phosphoinositide at the plasma membrane is PI(4,5)P₂, which has an important regulatory role towards the actin cytoskeleton dynamics. In addition to PI(4,5)P₂, also PI(3,4,5)P₃ is mainly

Figure 1. Definition of different plasma membrane curvatures. Plasma membrane can be flat and display ‘zero’ curvature. In cell protrusions, such as filopodia, the plasma membrane bends outwards generating negative membrane curvature, but also positive membrane curvature at the neck region. In contrast, inwards budding vesicles generate a large area of positive membrane curvature, but some parts of the neck regions also display negative membrane curvature.
found at the plasma membrane where it has multiple important tasks. (Di Paolo and De Camilli, 2006; Saarikangas et al., 2010). In certain cells, such as epithelial cells, and during specific cellular processes e.g. cell division, the distributions of PI(4,5)P₂ and PI(3,4,5)P₃ are segregated to distinct compartments at the plasma membrane (Saarikangas et al. 2010). During cytokinesis, PI(4,5)P₂ is localized to the contractile ring, whereas PI(3,4,5)P₃ is found at the poles of the two daughter cells (Janetopoulou and Devreotes, 2006).

In polarized epithelial cells, PI(4,5)P₂ has been found to preferentially localize to the apical surface, whereas PI(3,4,5)P₃ was found in the basolateral face (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). Local high concentrations of phosphoinositides e.g. PI(4,5)P₂ have been reported at the sites of active membrane remodelling, such as phagocytic sites and membrane ruffles (Botelho et al., 2000; Coppolino et al., 2002; Ling et al., 2006). These different spatial localizations of phosphoinositides can be achieved by concerted actions of certain PI kinases and phosphatases such as phosphoinositide-3-kinase (PI3K) and phosphatase and tensin homolog (PTEN), respectively. For example, during chemotactic cell motility of amoeba Dictyostelium discoideum, PI(3,4,5)P₃ is rapidly produced at the leading edge by PI3K and hydrolysed elsewhere by PTEN (Van Haastert and Veltman 2007). The high and polarized concentration of PI(3,4,5)P₃ at the leading edge is though to ensure that the reorganization of the actin cytoskeleton, which takes place downstream of PI(3,4,5)P₃, is correctly localized to generate the necessary force for directed cell motility.

Phosphoinositides are important regulators of the actin cytoskeleton. For example, different studies have demonstrated that sequestration of PI(4,5)P₂ leads to defects in the actin cytoskeleton and that forced increase of PI(4,5)P₂ concentration at the plasma membrane promotes actin filament assembly (Rozelle et al., 2000; Yamamoto et al., 2001; Raucher et al., 2000). The effects of phosphoinositides towards the actin cytoskeleton are thought to partially arise from the recruitment of proteins that activate Rho GTPases (GEFs) or proteins that inactivate Rho GTPases (GAPs). GEFs and GAPs are important regulators of RhoGTPase signaling pathways (see chapter 1.5). Importantly, phosphoinositides can also interact directly with numerous different actin-binding proteins, such as ADF/cofilins and WAVE/WASP proteins, and these interactions can either activate or inactivate proteins and/or regulate their sub-cellular localization (Saarikangas et al., 2010).

1.3. The Cytoskeleton

As the name implies, the cytoskeleton is maintaining cell shape by acting as a structural scaffold beneath the plasma membrane. However, this structural function of the cytoskeleton is only a sub-plot of the whole story. In fact, the cytoskeleton is a highly dynamic machinery, which can be rapidly reorganized to generate mechanical force to carry out many different tasks, ranging from cell division to nutrient uptake. The dynamic nature of these cellular scaffolds is made possible by the molecular nature of the cytoskeletal proteins, which can be rapidly assembled and disassembled as they were Lego brigs. In eukaryotic cells, the cytoskeleton is composed of three distinct assemblies: microtubules, intermediate filaments and actin filaments (also referred as microfilaments) (Bray, 2001).
Microtubules are long rigorous filaments, composed of two homologous GTP-binding proteins, α- and β-tubulin that form heterodimers. These dimers polymerize to form polar, cylindrical filaments that have a hollow cavity. The nucleation of microtubules takes place in microtubule organizing centres (MTOCs), and the microtubule dynamics can be adjusted by a large group of microtubule-associated proteins (MAPs). Besides providing structural support for the cell, microtubules are applied as tracts along which motor proteins such as kinesin and dynein can unidirectionally move and transport cargo. During cell division, microtubules are responsible for the segregation of the sister chromatids (Bray, 2001).

Intermediate filaments (IF) represent another class of cytoskeletal assembly. There are at least 65 genes that encode for IF proteins in humans and these are expressed in a cell type specific manner. Mutations in IF genes have been strongly linked to different human diseases ranging from cardiomyopathy to skin blistering disorders and progeria (Eriksson et al., 2009). The intermediate filament genes can be subdivided into five distinct classes that encode for fibrous proteins, which form dimeric coiled-coil complexes. These complexes are assembled into elastic networks that provide structural rigidity for cells and cell organelles (Herrmann et al., 2007). More recently, IF proteins have also been found to have non-mechanical roles, for example, in cell signaling pathways (Kim and Coulombe, 2007).

### 1.4. The Actin Cytoskeleton

Due to its high cellular concentration, actin is one of the most abundant protein molecules on earth and the importance of actin for vast number of different cellular processes is well established (Pollard and Cooper, 2009). Actin is highly conserved within species, which is demonstrated by the existence of bacterial actin-like molecules ParM and MreB, and the fact that yeast and rabbit actins are 88 % identical to each other at the amino acid sequence level (Erickson, 2007). The fact that actin molecule has remained highly conserved in evolution implies that the divergence in actin-driven cellular processes has been achieved through the evolution of effector molecules, which can precisely place and control actin dynamics at different scenes of action.

Structurally, actin is a globular molecule composed of four lobes with a central cleft occupied by a nucleotide, either ATP or ADP. The thing that makes actin so remarkable lays in its capacity to oligomerize into filaments and thus produce force. This can be achieved by the assembly of actin monomers (G-actin) into filaments (F-actin) (Figure 2). This cycling phenomenon between the two different forms (G-actin\(\rightleftharpoons\)F-actin) is taking place all the time in cells, and under proper ionic conditions, in a test tube. When incorporated into filaments, all actin monomers are facing the same direction and display different structural surfaces on each end, hence actin filament is a polar structure.

There are several biochemical characteristics that govern the transition of actin from monomers into oligomers. Actin monomers are preferentially added in ATP-bound stage and the addition favours one end of the protofilament, called the barbed (+) end. The ATP becomes rapidly and irreversibly hydrolysed to ADP and Pi once in the filament. As the phosphate (Pi) dissociates from the actin cleft, there is a slight structural change, which makes the filament more unstable and favours dissociation of monomers i.e. depolymerisation from the other end of the filament, known as the pointed (-) end (Pollard, 1986). The critical concentration for polymerization in vitro at the barbed end is 0.1 \(\mu\)M whereas in the pointed end it is 0.7 \(\mu\)M. In between these two
consistently growing from the barbed- and shrinking from the pointed end (Pollard, 1986; Wegner, 1982; Pollard and Weeds, 1984). This ATP powered cycle is collectively known as actin treadmilling (Figure 1). While this process is relatively slow in a test tube, in cells, the treadmilling is believed to be ~600 times faster. This difference can be explained by the promotion of actin dynamics by a huge amount of different actin-binding proteins that exist in cells (Pollard and Borisy, 2003).

1.5. Regulation of the actin cytoskeleton by the Rho GTPases

The Rho GTPases constitute a group of 20 signaling proteins that are involved in regulating numerous different cellular processes (Aspenström et al., 2007; Jaffe and Hall 2005). These proteins can be further divided into typical and atypical Rho GTPases (Aspenström et al. 2007). The typical Rho GTPases, which include e.g. Cdc42, Rac, RhōA and Rif are bound by either GDP or GTP. When active, the small GTPases contain a GTP molecule and hydrolysis of the GTP to GDP leads to conformational change in the protein structure that inactivates its signaling function. Since Rho GTPases usually have robust downstream effects, their nucleotide bound status needs to be accurately and tightly controlled. For this, cells have proteins, which control the cycling of GTPases between the inactive and active forms. The guanosine nucleotide
exchange factors (GEFs) catalyze the nucleotide exchange from GDP to GTP whereas the GTPase-activating proteins (GAPs) promote the hydrolysis of GTP to GDP (reviewed in Jaffe and Hall 2005). The activities and localization of GAP- and GEF-proteins are regulated by upstream signals and other regulatory proteins that specify their functions. For example, the activity of Rac GEF protein Vav is regulated by phosphoinositide signaling (Das et al., 2000). The atypical RhoGTPases, which include for example RhoH and RhoBTB, do not seem to cycle between different nucleotide-bound stages, rather, the activities of these proteins are regulated at the level of expression and through different binding-partners (Aspenström et al., 2007). Most members of Rho GTPase family have a robust effect on the organization of the actin cytoskeleton, which can very, depending on the GTPases, from induction of filopodia formation to formation of actin stress fibers (Aspenström et al., 2004). The best characterized for their effects towards the actin cytoskeleton are RhoA, Rac1 and Cdc42.

The small GTPase RhoA is linked to the regulation of cytokinesis, cell blebbing (discussed in chapter 2.1.2), actin stress fibres and focal adhesion complexes. Rac1, on the other hand has a pronounced role in activating pathways that lead to the formation of lamellipodia and membrane ruffles (discussed in chapter 2.1.1), which drive cell locomotion during developmental as well as pathogenic processes such as cancer cell invasion. The third well characterized Rho GTPase Cdc42 is a known inducer of filopodia formation (discussed in chapter 2.1.3). In addition, Cdc42 has a critical role in maintaining/promoting cell polarity in various organisms (Jaffe and Hall, 2005; Heasman and Ridley, 2008).

1.6. Regulation of actin dynamics by actin-binding proteins

The diversity of cellular functions is in many cases accomplished through copying and modifying the existing theme. This is also the case with many actin-binding proteins that regulate distinct actin-driven processes since many of them are derived from the same ancestral protein folds. For example, the ADF-H (actin depolymerisation factor homology) and WH2- (WASP homology-2) folds are found in high numbers in functionally divergent proteins. The diversity in function is acquired through small modifications into the existing fold and/or combinations of different domains in the context of the full length protein (Dominguez, 2007; Lappalainen et al., 1998).

Over a decade ago, ground-braking studies were made to reveal the minimal set of actin-binding proteins, which is required for actin-based motility in vitro. The result was somewhat surprising, since already at that time a huge number of actin-binding proteins had been identified. However, these studies found that for actin-based motility in vitro, only activated Arp2/3 complex, profilin, ADF/cofilin, and capping protein are required (Loisel et al., 1999). In the following chapters, these most fundamental regulators of actin dynamics are briefly discussed. It is important to note however, that in the more complex and challenging cellular surroundings, a significantly larger number of actin-binding/associated proteins are required to regulate actin dynamics (Figure 3).

1.6.1. Actin nucleation and elongation factors

Because the formation of an actin nuclei is the rate limiting process for efficient formation of actin filaments, and as the actin nuclei as such is unstable, cells need to establish ways to overcome this barrier in order to achieve efficient actin
Figure 3. The actin cytoskeleton is tightly regulated in cells by a vast number of actin-binding proteins. The WASP/WAVE-family of actin nucleation promoting factors activate Arp2/3-complex, which brings together seed of actin monomers resulting in the growth of a new actin branch from the side of the pre-existing "mother" filament. ATP-bound actin monomers associate to the barbed end of actin filaments. Once in the filament, the ATP in the monomer gets rapidly hydrolyzed into ADP, which makes the filament more unstable. The filament lengths are kept relatively short partially through the action of capping protein, which blocks the growth of actin filaments by binding tightly to the barbed end. Filaments can be linked together by actin crosslinking/bundling proteins such as α-actinin of fascin. ADF/Cofilins function near the pointed ends of actin filaments where they depolymerize and sever actin filaments thus increasing the amount of free actin monomers. Once detached from the filaments, ATP-actin monomers are bound by actin sequestering proteins such as β-thymosins or are bound by profilins, which catalyze the nucleotide exchange in the monomer from ADP-to ATP and thus make the monomers available for incorporation to the growing barbed ends for a new cycle of polymerization.
polymerization when and where necessary. For this, cells have a variety of actin nucleation factors that can bring together the necessary actin seed for the initiation of rapid polymerization. Cells can utilize several different pathways and protein components for actin filament nucleation and elongation. Currently, however, only two distinct protein components facilitate these actions towards actin: the WASP-homology domain-2 (WH2) and formins (Dominguez, 2009; Chesarone and Goode, 2009).

1.6.1.1. WH2-domain mediated filament nucleation/elongation

All actin nucleation factors that have been identified so far with the exception of formins make use of WH2 domains for their interactions with actin (Dominguez, 2009). WH2 is a small domain that is composed of only 17-27 amino acids. It folds into a helix, which binds between actin subdomains 1 and 3 and the helix is followed by a conserved extension containing the canonical LKKT-motif (Hertzog et al., 2004; Chereau et al., 2005) (Figure 3). The WH2 domain is found in various different proteins and thus associated with many functions. In actin nucleation factors, it is often found in tandem of 3-4 repeats, which facilitate the formation of actin filament nucleus needed for polymer assembly (Quinlan et al., 2005; Ahuja et al., 2007). Interestingly, it was recently shown that already one WH2-domain is sufficient for actin filament nucleation by TARP, a T3SS secretion system protein from Chlamydia trachomatis. It seems likely that TARP brings the actin nucleus together by forming oligomers (Jewett et al., 2006). Also, the muscle cell-specific actin filament nucleator leiomodin contains only one WH2 domain. In addition to its WH2 domain, leiomodin has two tropomodulin-like actin binding domains, which together with the WH2 can organize 2-3 actin monomers together to generate a seed for efficient actin polymerization (Chereau et al., 2008).

Another example, how the WH2 fold is utilized to promote actin polymerization is found in a group of proteins that are collectively called the nucleation promoting factors (NPFs). In mammals, there are many NPF-proteins including WASP, N-WASP, WAVE 1-3, WHAMM, WASH and JMY. In addition to these, there are bacterial proteins that mimic the action of mammalian NPFs such as Listeria monocytogenes ActA (Linardopoulou et al., 2007; Goley and Welch, 2006; Campellone et al., 2008; Zuccher et al., 2009; Takenawa and Suetsugu, 2007; Gouin et al., 2005). These proteins are needed to activate the Arp2/3 complex, which is one of the best known actin nucleation factors. The Arp2/3 complex is composed of seven subunits including two actin related proteins (Arp2 and Arp3). Alone, the Arp2/3 has a very poor nucleation activity (Mullins et al., 1998). However, in the presence of NPFs, which bring additional actin molecules as well as induce a conformational change in Arp2/3 structure, the actin nucleation activity of Arp2/3 is greatly enhanced. The activated Arp2/3 complex can nucleate new actin filaments at ~70° angles from the side of a pre-existing “mother” filament (Blanchin et al., 2000; Amann and Pollard, 2001) or at the barbed end of the mother filament (Boujemaa-Paterski et al., 2001; Pantaloni et al., 2000) to generate a branched, dendritic network of actin filaments. The existence of this branched network in cells, however, remains controversial (Svitkina and Borisy 1999; Urban et al., 2010).

In addition to participating in actin filament nucleation, WH2 domains can regulate actin dynamics by other means. For example, WH2 domains are found in proteins that function as actin filament barbed end elongation factors such as Ena/VASP proteins. These molecules can promote actin filament barbed end growth by acting as uncapping molecules...
that prevent the association of capping protein into filament barbed ends (Bear et al., 2002; Barzik et al., 2005). In addition, these proteins are expected to increase local actin monomer pools in the vicinity of filament barbed ends (Breitsprecher et al., 2008). Although rigorous effort has been made by numerous laboratories to elucidate the exact role(s) of Ena/VASP proteins towards actin, so far, no clear consensus has been reached (Bear and Gertler, 2009).

Intriguingly, the WH2 domain fold is also utilized in an opposite manner, to inhibit actin polymerization. β-thymosins are tiny ATP-actin binding proteins, which are composed almost solely of the WH2 domain (Paunola et al., 2002) and are thus efficient actin polymerization inhibitors by sequestering free actin monomers. Importantly, the structure of thymosinβ4 revealed an extended motif, which follows the canonical actin binding LKKT-motif. This extension binds between actin subdomains 2 and 4 and caps the pointed end of actin monomer thereby preventing actin polymerization (Irobi et al., 2004; Hertzog et al. 2004) (Figure 3). Thus the simple WH2-fold is utilized to regulate actin dynamics in very different ways.

1.6.1.2. Formin mediated filament nucleation/elongation
Formins constitute another family of actin nucleation/elongation factors. They are large (120-220 kDa) multidomain proteins, encoded by 15 distinct genes in mammals. Formins are composed of formin homology (FH1 and FH2) domains, which regulate formins’ actions towards actin. These domains are accompanied by regulatory regions, which are believed to specify the spatio-temporal activities of different formins. Formins seem to form autoregulated dimers that have two major roles in actin dynamics: promoting de novo actin filament nucleation and functioning as actin filament elongation factors by promoting processive barbed end elongation (Revived in Chesarone et al., 2010).

1.6.2. Actin filament capping by heterodimeric capping protein
Capping protein is a ubiquitously expressed protein that binds to actin
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filament barbed ends where it - truthfully to its name - functions as a cap to prevent the addition and loss of actin monomers. Capping protein is heterodimeric and composed of \( \alpha \) and \( \beta \) subunits (Cooper and Sept, 2008). Because capping protein has a very profound effect on actin dynamics, it must be tightly regulated in cells. There are many proteins that regulate its activity. One of the most important regulators is CARMIL, which binds capping protein with a high affinity and inhibits its binding to barbed ends (Yang et al., 2005). It is important to note that in addition to heterodimeric capping protein, there are several other actin-binding proteins such as Eps8, gelsolin and twinfilin, which cap actin filament barbed ends and can substitute capping protein in the in vitro actin motility assays (Dianza 2004; Sun 1999; Helfer et al. 2006).

1.6.3. Profilin recharges actin monomers

In mammals, there are four isoforms of profilin: ubiquitous profilin-1, brain-specific profilin-2 and poorly described testis-specific profilins III and IV (Birbach, 2008). In vitro, profilin has three known actions on actin dynamics. First, it can increase the nucleotide exchange on actin monomers from ADP to ATP by 1000-fold as compared with the exchange rate by simple diffusion. Secondly, it can sequester actin monomers, and thirdly, it can interact with the actin filament barbed end and feed it with ATP-actin monomers (Witke, 2004). In cells, profilin associates with numerous other proteins and \( \text{PI}(4,5)^3\text{P}_2 \), which regulate its activities. Profilin has been found to interact with actin polymerization/elongation factors such as N-WASP (Suetsugu et al., 1998), formins (Watanabe et al., 1997) and VASP (Reinhard et al., 1995) as well as many other actin regulatory proteins (Witke, 2004).

1.6.4. ADF/cofilins depolymerize and sever actin filaments

ADF/cofilin protein family consists of three members in mammals: Cofilins 1-2 along with ADF. Of these, cofilin-1 and ADF have relatively ubiquitous expression patterns whereas cofilin-2 seems to be mainly expressed in striated muscle cells (Vartiainen et al., 2002). These proteins have a well-characterized role in promoting actin filament treadmilling, which is mediated by cofilin’s actin filament depolymerizing and severing activities. The actin filament depolymerizing activity maintains high actin monomer pools in the vicinity of the barbed ends whereas the severing activity is thought to supply new actin filament barbed ends and thereby circumvent the nucleation problem (Carlier et al., 1997; Kiuchi et al., 2007; Lappalainen and Drubin, 1997; Ghosh et al., 2004; Andrianantoandro and Pollard, 2006; Pavlov et al., 2007). The activities of ADF/cofilins in cells are regulated by interactions with \( \text{PI}(4,5)^3\text{P}_2 \), other proteins, phosphorylation and pH (van Rheenen et al., 2007; Eiseler et al., 2009; Van Troys et al., 2008).

1.7. BAR protein superfamily mediates interactions between the actin cytoskeleton and plasma membrane

For some time, it has been known that roughly the same set of molecules regulate actin dynamics in both the formation of cell invaginations and cell evaginations i.e. cell protrusions. However, the specific details how the actin polymerization machinery is harnessed to drive these reversed processes have remained poorly understood. The discovery of BAR (Bin-Amphiphysin-Rvs) proteins as important regulators of endocytic events gave a big piece to this puzzle as these proteins could both sense/generate membrane curvature and link this activity directly to the recruitment of the actin polymerization
## Table 1. Different classes of BAR domains and their cellular functions.

<table>
<thead>
<tr>
<th>BAR Domain</th>
<th>Side-view</th>
<th>Examples of proteins</th>
<th>Examples of cellular processes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAR (Bin-amphipathic-RV/SH)</td>
<td><img src="image1.png" alt="BAR Diagram" /></td>
<td>Arfaptins, Tuba</td>
<td>Endocytosis (AMPA receptor) (Lu and Ziff 2005). Membrane ruffling (Kovacs et al. 2006).</td>
</tr>
<tr>
<td>I-BAR (Inverted-BAR, also known as IMD)</td>
<td><img src="image6.png" alt="I-BAR Diagram" /></td>
<td>MIM, ABBA, IRS5/3</td>
<td></td>
</tr>
<tr>
<td>MIM (pdb:2D1L)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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machinery. These BAR domain containing proteins have been placed in a myriad cellular events where membranes are remodeled (Suetsugu et al., 2009; Frost et al., 2009; Gallop and McMahon, 2005) (Table 1), and represent the largest and most diverse group of membrane deforming proteins.

Different BAR domains share structural similarities. They are composed of two α-helical monomers that dimerize in an antiparallel manner to form the elongated membrane binding/deforming BAR module. Based on structural differences, BAR domains can be further divided into three diverse sub-groups: the BAR, F-BAR and I-BAR domains (Frost et al., 2009; Gallop and McMahon, 2005). The diversity within these three subgroups arises from the numerous other protein/lipid-binding or enzymatic modules that are found in these proteins (See Figure 5). These other modules function to localize BAR proteins or to recruit other proteins to the sites of membrane remodelling (Suetsugu et al., 2009).

BAR domains have two mechanistically distinct ways of interacting with membranes. The general mechanism of membrane binding, which all BAR domains share, involves positively charged residues that span on one side of the dimeric BAR module. These residues are enriched at the distal ends of the dimer and facilitate the interactions with the negatively charged phospholipid headgroups of the membranes. In addition to electrostatic interactions, some BAR domains can insert amphipathic motifs into the membrane bilayer. These motifs are thought to enhance the binding affinity and they also increase the degree of membrane curvature (Gallop et al., 2006). Recent work using specific curvature-sensing assays suggested that these inserting motifs, rather than the intrinsic curvature displayed by the BAR domain, are responsible for membrane curvature sensing (Bhatia et al., 2009b). How these results account for the BAR domains that do not contain membrane inserting motifs remains elusive.

The binding of BAR domains to membranes is most likely a co-operative event, where the binding of one molecule facilitates the binding of the next. This results in the formation of rigid scaffolds that can mould the membrane according to the curvature of the protein domain. The degree of membrane curvature imposed by different BAR domains has been shown to correlate with the degree of curvature displayed by the domain structure, although variability exists as a result of tilting of the protein array relative to the membrane tubule axis and due to membrane inserting motifs (Gallop et al., 2006; Frost et al., 2008; Wang et al., 2009). The degree of intrinsic curvature in BAR domains has suggested to be involved in sensing specific membrane curvature i.e. BAR domains are recruited to sites in cells that display specific degree of membrane curvature (Peter et al., 2004). BAR domains can also exist in combination with other lipid-binding motifs such as PH- or PX-domains. In BAR-PH module containing SNX9 protein, such combination may provide additional specificity towards certain lipid species. In addition, the flexibility of this module may provide a means to change the degree of intrinsic curvature to allow variability for membrane curvature sensing and thus facilitating binding to different sized vesicles (Wang et al., 2008).

Interestingly, it was recently shown that the activity and efficiency of actin polymerization can be optimized in a membrane curvature-dependent manner. It was demonstrated that actin polymerization via N-WASP-WIP complex is enhanced by F-BAR proteins Toca-1 and FBPI7, which presumably place the polymerization complex in an optimal conformation by bending the membrane (Takano et al., 2008). As such, this study opened up new avenues for understanding
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how different BAR proteins function. Because BAR proteins act at the interface between plasma membrane and the actin cytoskeleton, they can bring together large protein complexes to curved membranes thereby providing an optimal launching pad for the initiation of force production that drives the formation of plasma membrane protrusions or invaginations.

1.7.1. BAR/N-BAR Domains
It has been over a decade since the first demonstration of the BAR domain to act as a membrane deforming unit was published (Takei et al. 1999). However, although the first crystal structure of an BAR domain was solved a couple of years later (Tarricone et al., 2001), it took many more years before the structure-function relationship of these domains as membrane curvature sensors/generators was fully appreciated (Peter et al., 2004). The BAR/N-BAR domains are croissant-shaped molecules that bind the membranes via their concave face. The concave face contains positively charged residues that are critical for the membrane binding by interacting with negatively charged lipid headgroups (Peter et al., 2004). A sub-set of the BAR domains (N-BARs) also contain hydrophobic motifs that can penetrate into the membrane bilayer, strengthening their interaction with membranes (Gallop et al., 2006; Masuda et al., 2006). Because these amphipathic α-helices of N-BAR domains only penetrate through one layer of the membrane bilayer, they are expected to induce membrane curvature through generation of bilayer asymmetry.

Proteins containing a BAR domain are known to be crucial regulators of endocytic events. This is achieved by their capacity to induce plasma membrane invaginations and simultaneously recruit the actin polymerization machinery to endocytic sites by interacting with Arp2/3 activators such as N-WASP (Kovacs et al., 2006; Otsuki et al., 2003; Shin et al., 2008; Ferguson et al., 2009). BAR/N-BAR domain containing proteins have also been liked to several other cell biological processes such as muscle t-tubule biogenesis, membrane ruffling, podosome function and regulation of mitochondrial and autophagosomal membrane dynamics (reviewed in Frost 2009, Takahashi 2009) (Table 1).

1.7.2. F-BAR/IF-BAR
The F-BAR (FCH and BAR) module was first discovered as a conserved domain found in proteins that were localized to the sites of actin remodelling (Aspenstrom, 1997). Later on, they were established as a functional sub-group of BAR domains as they were shown to bind and deform negatively charged membranes and to induce membrane invaginations in cells (Itoh et al., 2005). The crystal structures of F-BAR domains revealed an α-helical dimeric bundle, which was more elongated and displayed a shallower degree of curvature compared with the BAR domain structure (Henne et al., 2007; Shimada et al., 2007). Due to these structural differences, F-BAR domains deform membranes into tubules that are generally wider in diameter than those induced by BAR domains, although membrane tubules induced by F-BAR domains have been found to display variability in their diameters due to tilting of the F-BAR domain scaffold relative to the tubular axis (Frost et al., 2008; Wang et al., 2009; Henne et al., 2007; Shimada et al., 2007). The F-BAR domain of pacsin/syndapin contains potential insertion loops in its concave face, suggesting that some F-BAR domains may insert into the membrane bilayer although this remains to be experimentally demonstrated (Wang et al., 2009). Recently, the crystal structure of the full-length F-BAR protein pacsin/syndapin was solved. Interestingly, this structure revealed that the membrane binding surface of the F-BAR interacts with the C-terminal SH3 domain. Biochemical and cell biological analyses demonstrated that this interaction inhibited the
membrane deformation activity of pacsin/syndapin. Importantly, addition of the SH3 domain ligand (polyproline sequence of dynamin) was sufficient to release this autoinhibition and activate the membrane deformation activity of the F-BAR domain. As many BAR, F-BAR and I-BAR proteins contain a SH3 domain, this study might provide a more general mechanistic view on how the membrane binding/deforming activity of different BAR proteins could be regulated in cells (Rao et al. 2010).

In many cases, F-BAR proteins have been associated to endocytic events (Itoh et al., 2005; Shimada et al., 2007; Toguchi et al. 2010; Henne et al. 2010). A recent study analyzed the phenotypes of C.elegans nematodes where both Toca-1 and Toca-2 genes were inactivated. This study demonstrated that Toca genes are important for clathrin-mediated endocytosis and that they are genetically linked to the same pathway as N-WASP and WAVE-proteins (Giuliani et al., 2009). Toca protein has been shown to activate Arp2/3 mediated actin polymerization by releasing N-WASP autoinhibition (Ho et al., 2004). Moreover, this Arp2/3 activation was recently shown to be membrane curvature dependent, providing maximal actin polymerization rates at the highest membrane curvatures (Takano et al., 2008). Similarly to Toca, F-BAR protein Cip4 has been strongly implicated in endocytosis. Cip4 knockout mice have an endocytic phenotype and displayed lower post-prandial glucose levels due to altered plasma membrane expression of GLUT4. Moreover, fibroblasts extracted from Cip4 knockout mice displayed decreased fluorescein dextran, horseradish uptake and transferring uptake as compared to wild type cells (Feng et al., 2009). These results are corroborated by RNAi mediated knockdown experiments of Cip4 performed in mouse embryonic fibroblasts. These experiments showed that Cip4 knockdown results in delayed platelet derived growth factor receptor-β internalization and consequent increase in the formation of dorsal ruffles (Toguchi et al., 2010). Interestingly, Syp1, which is an F-BAR protein found in budding yeast, was recently demonstrated to participate in early events of endocytosis where it, in sharp contrast to Toca, acted as an inhibitor of Arp2/3 complex by interacting with Las17/WASP, possibly serving as a timer for endocytic events (Boettner et al., 2009). The most compelling evidence for the role of F-BAR proteins in endocytosis has been recently presented by Henne and co-workers, who demonstrated that the F-BAR proteins FCHO1/2 are actually prerequisites for the budding of clathrin-coated vesicles. These results suggested that the F-BAR domain of FCHO-proteins deforms the plasma membrane to make the initial clathrin-coated bud and subsequently recruit clathrin-adaptor proteins to these sites via its other domains to form the clathrin-coated vesicle (Henne et al. 2010). Together, these results suggest that different F-BAR proteins play multiple and partially overlapping roles in orchestrating endocytic events.

Although initially all F-BAR proteins were linked to the formation of membrane invaginations, recent findings suggest that a sub-set of the F-BAR domains could also participate in the formation of cell protrusions. The Takenawa group recently identified two F-BAR domain containing proteins Fes and Fer as important molecules for lamellipodia formation and cell migration in mammalian cells (Itoh et al., 2009). Also, the over-expression of F-BAR protein pacsin/syndapin in mammalian cells has been shown to induce the formation lamellipodia and filopodia. Furthermore, it was shown by RNAi experiments that that pacsin/syndapin is required for neuronal arborisation (Dharmalingam et al., 2009). Similarly, in Drosophila melanogaster, pacsin/syndapin was shown to promote the expansion of postsynaptic membrane systems (Kumar et al., 2009).
evidence for the inverse function of some F-BAR proteins was obtained by Guerrier and colleagues who demonstrated that the F-BAR domain of SrGAP2 protein deforms membranes in opposite direction as other F-BAR proteins tested so far. In line with their \textit{in vitro} results, it was shown that srGAP2 induces filopodia-like protrusions in cells and is important for neuronal branching (Guerrier et al., 2009). How this is achieved mechanistically remains to be shown and would ideally require a crystal structure of this inverse-F-BAR (IF-BAR) domain.

1.7.3. I-BAR Domains

I-BAR domain was originally discovered as a conserved domain that resides in the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Different BAR proteins are composed of variable combinations of functional domains that specify their cellular functions. SH3: Src homology 3-domain, PX: PX domain, PH: PH domain, PPP: proline rich extension, ArfGAP: ArfGAP domain, PTB: PTB domain, PDZ: PDZ-binding domain, RhoGEF: RhoGEF domain, RhoGAP: Rho GAP domain, HR1: Rho effector or protein kinase C-related kinase homology region 1 homologue, Ank: Ankyrin domain, WH2: Wiskott-Aldrich homology 2 domain, Tyr-kinase: tyrosine kinase domain, CRIB: Cdc42/Rac interactive binding domain.}
\end{figure}
N-terminal regions of five mammalian proteins. These included IRSp53 and missing-in-metastasis (MIM) proteins and therefore this domain was initially named as IMD (IRSp53/MIM homology domain) (Yamagishi et al., 2004). In evolutionary terms, I-BAR domain fold is ancient as it is present in organisms like C. elegans and Dictyostelium discoideum. Through gene duplications, the I-BAR family has expanded in evolution and the five mammalian I-BAR members can be further divided into two subgroups: the MIM subfamily and the IRSp53 subfamily (Figure 6), (Scita et al., 2008) (II, Fig.3).

1.7.3.1. IRSp53
IRSp53 (Insulin Receptor Substrate p53) is the most intensively studied member of I-BAR proteins, however its cell biological role has remained somewhat elusive. IRSp53 has four major isoforms that are generated through alternative splicing. The longest L-form contains a C-terminal WH2 domain and is mainly expressed in the brain. The S-form is also expressed in the brain and is characterized by a C-terminal PDZ (post synaptic density) binding motif (Scita et al., 2008; Okamura-Oho et al., 2001). IRSp53 has been shown to bind both Rac1 and Cdc42 GTPases in their GTP-bound, activated states. The Rac1 binding region is located in the I-BAR domain, whereas Cdc42 binds to the CRIB domain situated between the I-BAR and SH3 domains (Miki et al., 2000; Govind et al., 2001). Many of the known interactions of IRSp53 with other proteins are mediated through its SH3 domain. These binding partners include N-WASP (Lim et al., 2008), WAVE2 (Miki and Takenawa, 2002), Mena/VASP (Krugmann et al., 2001), mDia2 (Fujiwara et al., 2000) and Eps8 (Funato et al., 2004; Disanza et al., 2006). These binding partners are all associated with the regulation of the actin cytoskeleton, suggesting a critical role of the SH3 domain for the function of IRSp53. Interestingly, a recent study demonstrated that the SH3 domain is indeed important for the localization of IRSp53 to lamellipodia and that it is regulated by phosphorylation-dependent binding to 14-3-3-proteins. Binding of 14-3-3 to IRSp53 prevents the association of SH3 domain to its ligands and thus keeps the protein inactive (Robens et al., 2010). Another regulatory mechanism is mediated by interaction with Kank (kidney ankyrin repeat-containing protein), which inhibits the association of IRSp53 with Rac1 but not with Cdc42 (Roy et al., 2009).

In cells, IRSp53 has been reported to function at various structures, which all have a common nominator of rapid actin dynamics. The most validated role of IRSp53 is in the formation of filapodia and it is shown to localize to the tip of filapodia and seems to act downstream of Cdc42 signaling (Krugmann et al., 2001). IRSp53 has also been shown to play a crucial role in lamellipodial dynamics (Miki et al., 2000; Tsujita et al., 2006). In primary neurons, IRSp53 has been shown to localize to post-synaptic part of dendritic spines through interaction with PSD95, which is an abundant protein involved in the formation and regulation of excitatory synapses and dendritic spines. This study further demonstrated that RNAi mediated knockdown of IRSp53 reduced spine density (Choi et al., 2005). More recently, IRSp53 was demonstrated to play a role in epithelial tight junctions. It was shown that IRSp53 interacts with Lin-7 through its PDZ-binding domain, and that this interaction was important for the assembly of tight junctions (Hori et al., 2005; Massari et al., 2009).

The first knockout animals of I-BAR proteins were generated by targeting the IRSp53 gene. Two laboratories published their results around the same time with similar results. They demonstrated that only a portion of IRSp53 -/- mice developed into adulthood. However, the ones that were born, displayed no morphological abnormalities. Although
no morphological alterations were detected, the IRSp53 knockout mice displayed altered synaptic transmission, which resulted in behavioural defects and demonstrates the importance of IRSp53 for the correct function of the nervous system (Sawallisch et al., 2009; Kim et al., 2009).

1.7.3.2. IRTKS
In the initial characterization of this protein, Millard and co-workers demonstrated that IRTKS (Insulin Receptor Tyrosine Kinase Substrate) is an insulin receptor substrate that has ubiquitous expression pattern. They also provided biochemical evidence that IRTKS bundles actin filaments with its I-BAR domain and binds actin filaments through its C-terminal WH2 domain. It was also shown that IRTKS binds the small GTPase Rac1 through the I-BAR region. In cells, IRTKS was shown to induce protrusions composed of actin clusters, which had distinct appearance from those induced by IRSp53. The distinctive region between these homologous proteins was mapped to the C-terminal part of IRTKS (Millard et al., 2007).

Domain swapping experiments conducted by Ed Manser’s lab suggest that the differences between IRTKS and IRSp53 arise from differences in the SH3-domain, which seems to facilitate interactions with different binding partners (Robens et al., 2010). Interestingly, IRTKS can be hijacked by the bacterial pathogen Enterohemorrhagic Escherichia coli during the formation of bacterial pedestals (Vingadassalom et al., 2009).

1.7.3.3. FLJ22582
FLJ22582 is currently the only uncharacterized member of the mammalian I-BAR proteins. It has a similar domain organization as the IRSp53-branch and is composed of an I-BAR domain, a SH3 domain and a WH2-like extension (Scita et al., 2008).

1.7.3.4. MIM
MIM (MTSS1) was originally discovered as a gene, which expression is down-regulated in metastatic bladder cancer cell lines but is not down-regulated in non-metastatic bladder cancer cells (Lee et al., 2002). These findings raised interest towards this molecule and its potential role in metastatic transformation. However, so far the results regarding the role of MIM as a tumor suppressor have remained controversial (Wang et al., 2007a; Ma et al., 2007; Bompard et al., 2005; Parr and Jiang, 2009). MIM has a tissue specific expression pattern. During development MIM mRNA is expressed in the ventral part of the developing neural tube and also in the developing myocytes. In adult mice, expression of MIM is found in the kidney, liver and in the Purkinje cells of the cerebellum (Mattila et al., 2003). MIM gene is alternatively spliced in some tissues, however the consequences of the alternative splicing are poorly understood (Glassmann et al., 2007; Machesky and Johnston, 2007).

MIM protein is composed of N-terminal I-BAR domain (Yamagishi et al., 2004) and a C-terminal actin monomer-binding WH2 domain and variable regions in between these domains (Mattila et al., 2003; Woodings et al., 2003). MIM I-BAR domain binds the small GTPase Rac1 (Suetsugu et al., 2006; Bompard et al., 2005). The central region of MIM has several proline-rich extensions, which have been shown to facilitate the binding to the SH3 domain of cortactin (Lin et al., 2005). This interaction was shown to enhance cortactin-mediated actin polymerization in vitro. Furthermore, the central region of MIM contains several tyrosine residues that are phosphorylated when cells are stimulated with platelet derived growth factor (PDGF). This phosphorylation event is possibly mediated by Src kinase. Moreover, it was shown that the phosphorylation of MIM from Tyr-397
and Tyr-398 is important for its ability to induce dorsal ruffling in NIH3T3 cells (Wang et al., 2007b). The C-terminal WH2 domain of MIM binds actin monomers with a high affinity favouring ATP-bound monomers over ADP-bound (Mattila et al., 2003). MIM has also been found to be a Sonic hedgehog (Shh) responsive gene. It was proposed that MIM enhances Shh-pathway transcription through direct interactions with Gli1/2-transcription factors (Callahan et al., 2004; Gonzalez-Quevedo et al., 2005). Surprisingly, it was recently shown that the Drosophila MIM/ABBA homologue (dMIM) contributes to border cell migration by inhibiting endocytosis to result in more persistent guidance cue signaling. In molecular terms, this was achieved by competition between dMIM and pro-endocytic proteins endophilin and CD2AP for cortactin binding (Quinones et al. 2010).

Figure 6. The domain structures and known interaction partners of I-BAR proteins.
Although substantial efforts have been made to elucidate the cellular role of MIM, the results have remained somewhat inconclusive. Much of this is due to lack of suitable antibodies against MIM, which has compelled researchers to conduct their studies using over-expression constructs that do not allow far-reaching interpretations to be made.

1.7.3.5. ABBA
ABBA (Actin binding protein with BAIAP homology) displays high homology to MIM (~58% identical) and was originally named by Yamagishi and colleagues (Yamagishi et al., 2004). No experimental work was, however, presented in this study. In fact, the only study so far conducted on ABBA is presented in this thesis (Results, Section 4) and thus ABBA will not be further discussed here.

2. Protrusive cellular events involving membrane deformation and actin cytoskeleton remodeling

Cells form plasma membrane protrusions to facilitate important functions such as cell movement or the uptake of extracellular material. Although these events are regulated by various different signaling pathways, in many cases they make use of the same effector molecules, such as the Arp2/3 complex.

2.1. Cell migration and invasion

Cell migration and invasion is a crucial process for the proper development of mammals. Post-development, cell migration takes place, for example, during wound healing, and is also utilized by leucocytes to provide protective immunity. Inappropriate cell migration and invasion takes place in metastatic disease, where cancer cells detach from the primary tumor, break down tissue barriers and penetrate into blood and/or lymphatic vessels, through which they can circulate to different organs and establish secondary sites of tumors (metastases). There are several different modes of cell migration that are characterized by differences in the molecular pathways and which can be, at least partially, distinguished from each other based on differences in cell morphology and/or pattern of migration (reviewed in Friedl and Wolf, 2010). All cell migration events involve remodeling of the plasma membrane, which is facilitated by the force produced by the cytoskeleton (Bray, 2001).

2.1.1. Lamellipodium leads the way

Lamellipodium is a sheet-like structure, which is responsible for generating

Figure 7. Different protrusive cell structures found in eukaryotic cells. Blebs are plasma membrane expansions, which are associated with different cellular processes. Lamellipodia and filopodia as well as blebs are important for cell locomotion and found at the leading edge of migrating cells. Lamellipodium is composed of branched actin network whereas filopodia contain linear bundled actin. Phagocytosis and macropinocytosis are endocytic events regulating the uptake of certain extracellular particles. Invadopodia and podosomes are cell surface sites displaying proteolytic activity. Certain bacteria can induce the formation of actin rich protrusions called pedestals beneath their attachment sites in intestinal epithelia.
necessary protrusive force for cell locomotion. It is found at the leading edge of migrating cells that are cultured in two-dimensional (2D) surroundings. Lamellipodia has generally been considered a good model to study actin generated motility, and therefore exhaustive efforts have been made to understand this structure. Lamellipodia encompasses a branched array of actin filaments that are kept relatively short for optimal force generation (Mullins et al., 1998; Svitkina and Borisy, 1999). Recently, however, some labs have reported that in mature lamellipodia, actin filaments are not branched (Urban et al., 2010; Koestler et al., 2008; Resch et al., 2002). The available structural and biochemical data favour the branched model (Blanchoin et al., 2000; Volkman et al., 2001) however further studies are required to address these voices of concern. Furthermore, while most of the cell biological studies conducted so far have been addressing cell motility in 2D cell culture conditions, studies conducted in more physiological 3D environments such as collagen matrices or in tissues, imply that studying lamellipodia as a means of cell motility is one-sided and the conclusions that can be drawn from these results are not universally applicable to describe in vivo cell motility. This is because cells migrating in 3D have a somewhat different morphology as supposed to cells migrating in 2D. In 3D, lamellipodia are for the most part replaced by different type of protrusions: blebs, invadopodias, pseudopodias and filopodia-like spikes. Therefore, additional work needs to be done to bridge the gap of knowledge between 2D results in cell migration as supposed to the more physiologically obtained 3D results (Provenzano et al., 2009; Wolf and Friedl, 2009; Pampaloni et al., 2007).

The most pronounced and best characterized pathway leading to lamellipodia formation and membrane ruffling is through the activation of the small GTPase Rac1. Elegant study on photoactivatable Rac1 demonstrated that a precise local activation of Rac signalling is sufficient to generate cell protrusions and plasma membrane ruffling (Wu et al. 2009). Rac signaling activates WAVE-family proteins, which in turn can activate the Arp2/3 complex to generate typical lamellipodial actin meshwork (Takenawa and Miki, 2001; Hall, 2005). Rac signaling further enhances WASP/WAVE protein activity by increasing the amount of PI(4,5)P2, through the activation of PI5K (Saarikangas et al., 2010). Additionally, Rac1 activates PAK1 to result in a signaling cascade that increases the phosphorylation and inactivation of actin depolymerisation by ADF/cofilins (Edwards et al., 1999). In addition to WASP/WAVE family proteins, also another NPF, cortactin, has been linked in the formation of lamellipodia. It can activate and enhance Arp2/3-mediated actin polymerization and its activity is further enhanced by binding of other effectors such as MIM (Uruno et al., 2001; Kinley et al., 2003; Daly, 2004; Lin et al. 2005; Le Clainche and Carlier, 2008).

Interestingly enough, several BAR domain containing proteins have been liked in the formation/maintenance of lamellipodial structures, providing a direct link between cytosolic and cytoskeletal proteins in active membrane deformation. The I-BAR protein IRSp53 binds WAVE2 and was found to be important for lamellipodia formation in A431 human epithelial carcinoma cell line (Suetsugu et al., 2006). Also some F-BAR proteins have been shown to regulate lamellipodia formation. The extended F-BAR unit (hence named as FX) has recently been shown to be involved in lamellipodia formation through the activation of Fer kinase (Itoh et al., 2009).

It is worthy to note, that actin polymerization at the leading edge is not sufficient for efficient cell locomotion to occur. Cells need to be adhered to the
substratum and these cell-matrix adhesions need to be directly linked to the actin cytoskeleton. This is achieved via adhesive structures called focal adhesions that are enriched with trans-membrane adhesion molecules called integrins, which link the cytoskeleton to the cell substratum. This coupling ensures that the force generated by the actin polymerization machinery is transformed into formation of protrusions. In addition, motile cells need to form a trajectory between cell body and tail, which is facilitated by contractile actomyosin forces that are tightly coupled to focal adhesions (Le Clainche and Carlier, 2008; Hu et al., 2007; Mitchison and Cramer, 1996).

2.1.2. Blebs– dissolution of the holy marriage
Membrane blebs describe the events that occur upon local disruption of the linkage between plasma membrane and the underlying cortical actin cytoskeleton, which results in the rapid expansion of plasma membrane due to cells’ internal pressure. For a long time, blebs were mainly considered as a phenomenon related to apoptotic cell death, however, recent studies have placed these odd protrusions as important contributors in events such as cancer cell migration and viral infection (Sahai, 2005; Pinner and Sahai, 2008; Charras and Paluch, 2008; Mercer and Helenius, 2008).

Membrane blebs are usually a few micrometers in diameter and have a characteristic life-cycle of 1-2 minutes. The bleb life cycle is composed of nucleation, expansion and retraction phases (Charras and Paluch, 2008; Bovellan et al., 2009; Fackler and Grosse, 2008). Interestingly, in contrast to other cell protrusions, the formation of bleb does not require actin polymerization to drive the protrusion. However, after expansion slows down a new actin cortex is rapidly reassembled. ERM-family proteins such as ezrin have been suggested to facilitate the re-establishment of actin-membrane linkage through coexistent binding to PI(4,5)P₂ and actin filaments. In the last step, the recruitment of myosin is used to generate necessary contractile actomyosin structures to drive the bleb retraction by the RhoA-ROCK pathway (Charras and Paluch, 2008; Fackler and Grosse, 2008). ROCK (Rho-associated coiled-coil forming kinase) is a serine/threonine kinase and a downstream effector of RhoA that phosphorylates target proteins, which include the myosin light chain and the myosin-binding subunit of the myosin phosphatase. Together, these phosphorylation events increase myosin light chain phosphorylation and stimulate actin cross-linking by myosin resulting in increased actomyosin contractility (Narumiya et al., 2009). Although many key aspects of membrane blebs have been resolved during recent years, many uncertainties still lay as regards to the molecular mechanisms that regulate the three stages of membrane bleb life-cycle.

2.1.3. Filopodia
Filopodia are plasma membrane protrusions that have the resemblance of thin fingers. Filopodia are relatively short lived and the average life-time of filopodia has been reported to vary between 1-3 minutes, depending on the cell type (Ahmed et al., 2009). Filopodia serve as probes that sense the wonders of the extracellular world; tracking down guidance cues and adhesion points from the substratum. Some reviews have divided filopodia and microspikes as different structures based on their length and dynamics (Adams, 2001) and therefore some controversy exists in the nomenclature (Faix et al., 2009). Here, both of these dynamic F-actin rich structures are categorized as filopodia. The characteristic feature of filopodia is that they contain a dense core made of actin filaments. The actin filaments are usually tightly bundled and orientated so that their barbed ends are facing towards the plasma membrane.
at the filopodia tip (Faix and Rottner, 2006; Mattila and Lappalainen, 2008; Gupton and Gertler, 2007). The growth of actin filaments against the plasma membrane is considered to be responsible for generating the necessary force that drives the energetically unfavourable plasma membrane deformation from flat, sheet-like structures into filopodial tube-like structures. However, evidence is accumulating that also proteins that directly deform the plasma membrane, such as I-BAR and IF-BAR proteins, participate in filopodia formation (Ahmed et al. 2009; Guerrier et al. 2009). The dynamics of a given filopodium is regulated by a number of actin-binding proteins that regulate actin re-arrangements in the tip, shaft and/or base of the filopodia to give its characteristic features. The actin dynamics are mainly regulated by canonical actin-binding proteins, which also regulate many other filopodia-independent actin-based events (Faix and Rottner, 2006; Mattila and Lappalainen, 2008; Gupton and Gertler, 2007; Svitkina et al., 2003).

Currently, there are two GTPase molecules; Cdc42 and Rif that have well characterized roles in giving rise to filopodia by using at least partially different sets of downstream effector proteins. Of these, Rif has remained relatively poorly characterized GTPase, which seems to generate filopodia by activating the formin mDia2 (Pellegrin and Mellor, 2005). In contrast to Rif, Cdc42 is a rigorously studied signaling molecule involved in filopodia initiation. It is activating at least three independent pathways that can lead to filopodia formation. Cdc42 can activate Arp2/3-complex-mediated actin polymerization through the recruitment and activation of WASP-proteins (Rohatgi et al., 1999; Rohatgi et al., 2000; Machesky and Insall, 1998). However, the importance of the Cdc42-Arp2/3 mediated pathway in filopodia formation remains disputed since Cdc42 can give rise to filopodia in the absence of WAVE or Arp2/3 (Steffen et al., 2006). Independently of its activities towards Arp2/3 pathway, Cdc42 has also been shown to have other downstream effectors. These include mDia2 (Peng et al., 2003), a member of the formin family of actin nucleators that promote the formation of unbranched actin arrays (Chesarone et al., 2010). The binding of Cdc42 to the CRIB-motif of mDia2 is proposed to release the autoinhibition of mDia2 activating its actin-nucleation activity that promotes filopodia (Peng et al., 2003). Additionally, Cdc42 has been implicated as a regulator of IRSp53-mediated filopodia formation (Govind et al., 2001). Most likely, IRSp53 deforms plasma membrane outwards via its I-BAR domain and synergistically brings together a set of molecules that are responsible for the actin re-arrangements, which together give rise to filopodia (Ahmed et al., 2009). Supporting this view, it seems that both the SH3 domain and the intact I-BAR domain are necessary for the IRSp53 mediated filopodia formation (Lim et al., 2008). The IRSp53 interacting actin-regulatory proteins include, for example, Ena/VASP family member Mena (Krugmann et al., 2001) and actin-binding protein Eps8 (Disanza et al., 2006).

Filopodia are evolutionarily well conserved structures. The existence of filopodia in the highly mobile amoeba Dictyostelium discoideum has lead to extensive research on the role of filopodia in cell motility (Insall and Andrew, 2007). Filopodia are often found projecting from lamellipodia, although neither of these structures are prerequisites for the other. In metazoans, filopodia have been found to be of particular importance for many developmental processes, for example, during dorsal closure of Drosophila melanogaster (Martin-Blanco et al., 2000) and ventral closure of C. elegans (Williams-Masson et al., 1997). In mammals, filopodia are important for multiple aspects of neuronal cell physiology, regulating neurite initiation (Kwiatkowski et al., 2006). Independently of its activities towards Arp2/3 pathway, Cdc42 has also been shown to have other downstream effectors. These include mDia2 (Peng et al., 2003), a member of the formin family of actin nucleators that promote the formation of unbranched actin arrays (Chesarone et al., 2010). The binding of Cdc42 to the CRIB-motif of mDia2 is proposed to release the autoinhibition of mDia2 activating its actin-nucleation activity that promotes filopodia (Peng et al., 2003). Additionally, Cdc42 has been implicated as a regulator of IRSp53-mediated filopodia formation (Govind et al., 2001). Most likely, IRSp53 deforms plasma membrane outwards via its I-BAR domain and synergistically brings together a set of molecules that are responsible for the actin re-arrangements, which together give rise to filopodia (Ahmed et al., 2009). Supporting this view, it seems that both the SH3 domain and the intact I-BAR domain are necessary for the IRSp53 mediated filopodia formation (Lim et al., 2008). The IRSp53 interacting actin-regulatory proteins include, for example, Ena/VASP family member Mena (Krugmann et al., 2001) and actin-binding protein Eps8 (Disanza et al., 2006).
et al., 2007; Dent et al., 2007), neuronal branching, neurite outgrowth (Guerrier et al., 2009; Luo, 2002) and growth cone dynamics (Gallo and Letourneau, 2004). In addition, filopodia-like structures function as precursors of dendritic spines. Mature dendritic spines are mushroom shaped cell protrusions that function as the postsynaptic partners of most excitatory synapses and thus are essential for efficient synaptic transmission. The maturation process of dendritic spines involves an intermediate stage where the unmatured dendritic spine adopts a dynamic filopodia-like stage controlled by the small GTPase Rif through its effector mDia2, which is possibly involved in the generation of the spine shaft and/or finding the presynaptic partners (Sekino et al., 2007, Hotulainen et al. 2009). In addition to neuronal cells, filopodia are also found to be important for the formation of phagocytic cup in macrophages (Niedergang and Chavrier, 2004; Kress et al., 2007), during vessel branching (De Smet et al., 2009) and during the formation and maintenance of epithelial adhesions (Vasioukhin et al., 2000; Vasioukhin and Fuchs, 2001; Yamazaki et al., 2007).

Interestingly, many pathogens have developed strategies where they utilize filopodia as a means to infect neighbouring non-infected cells. Vaccinia virus has developed an elegant way of capturing the host cell cytoskeleton to facilitate its intracellular movement (Cudmore et al., 1995) and this mechanism is well conserved among vertebrate poxviruses (Dodding and Way, 2009; Rottner and Stradal, 2009). Vaccinia utilizes a viral protein A36R through which it assembles a protein complex that include Nck adaptor proteins, N-WASP and Arp2/3 complex to induce the formation of a actin comet tail that drives viral motility (Gouin et al., 2005; Frischknecht et al., 1999). Upon budding out of the cell, Vaccinia can remain attached to the plasma membrane and activate cytosolic actin comet tail resulting in the formation of filopodia that acts as an elevator for the virus, transporting it to the surface of the neighboring cell (Cudmore et al., 1995). Also Pseudorabies virus, African swine fever virus, Herpex simplex virus and Mardburg virus have been shown to induce filopodia upon infection. Although not much is now about the details, there seems to be a correlation between the number of filopodia and viral spread, indicating that these structures might be utilized in a to promote cell-cell transmission thereby enhancing the viral infection process (Levine et al., 1998; Jouvenet et al., 2006; Dixit et al., 2008; Oh et al., 2009; Kolesnikova et al., 2007).

Microvilli are finger-like protrusions found on the surface of absorptive epithelia of the intestine and kidneys where they increase the absorptive area. Also some non-absorptive cells such as lymphocytes have microvilli on their surface. Although microvilli have the general appearance of filopodia, it seems that the molecular architecture that builds these protrusions is at least partially distinct from that of filopodia. Microvilli are composed of parallel bundles of actin filaments that are cross-linked by fimbrin, epsin and villin and linked to the plasma membrane by ERM proteins. At the tip of microvilli, there is an electron dense mass (reviewed in (Higgs and Pollard, 2000).

The wide variety of different functions and molecules governing the formation of filopodia implies that there are in fact many different types of filopodia. These are crafted by different recipes of adaptor molecules to serve the specific needs of different cell types and different cellular processes. Thus, much work needs to be done to better understand why, when, where and how these differences are formed.

2.1.4. Podosomes and Invadopodia

Podosomes and invadopodia form a distinctive class of actin-driven protrusions as they are proteolytically active and
have a pronounced role in degrading the extracellular matrix through the activity of matrix metalloproteases (MMP's). These structures share many similarities with each other; for example, they are enriched with integrins and contain similar sets of regulatory proteins including Rho GTPases, actin nucleation promoting factors and Arp2/3 complex (Linder, 2007). In addition, phosphoinositide signaling has been suggested to regulate both podosomes and invadopodia (Chellaiah, 2006; Oikawa et al., 2008; Symons, 2008). However, there are several distinct features, which enable one to differentiate these structures. Generally, they exist in different cell types and display differences in their proteolytic activity, their persistence and number (Linder, 2007; Gimona et al., 2008).

Podosomes are found in various different types of mammalian cells. In osteoclasts, podosomes are structural components of the bone reabsorptive structures called the sealing zones and have an important role in bone remodelling. Podosomes are also found in endothelial cells where they are thought to participate in vessel wall remodelling. In addition to these, podosomes have been found in the cells of the immune system such as macrophages and dendritic cells (Gimona et al., 2008). Podosomes are small and found in large number of up to 100 per cell. They are relatively dynamic structures with the average life time of 2-12 minutes (Linder, 2007). Interestingly, tubular membrane invaginations have been shown to be associated with podosomes and the N-BAR domain proteins endophilin and amphiphysin are concentrated on these sites although currently their function there is ill defined (Kusumi et al., 2007; Ochoa et al., 2000).

Seminal work by the Chen laboratory originally identified invadopodia as matrix degrading adhesive structures in transformed chicken embryonic fibroblasts (Chen, 1989; Mueller and Chen, 1991). Invadopodia are found in invasive carcinoma cells and these structures are supposed to promote the invasion of metastic cells through extracellular barriers. In comparison with podosomes, invadopodia are larger in size and more persistent with a life time up to an hour. It seems that upon transformation into a cancer cell, the hunger gets bigger since invadopodia are found to be proteolytically more active than podosomes (Linder, 2007; Gimona et al., 2008). Recently, invadopodia have been found enriched in cholesterol, which might regulate the correct trafficking of proteases and other cargo to these sites (reviewed in (Caldieri and Buccione, 2009).

2.2. Cell-cell interactions and intercellular communication

In tissues, cells are for the most part surrounded by other cells and form adhesive and communicative structures with each other that maintain correct cell polarity and tissue integrity. The formation and maintenance of both communicative and adhesive structures involves dynamic interplay between the plasma membrane and the actin cytoskeleton. Interestingly, the formation and/or maintenance of these structures makes use of actin-based structures, such as filopodia and lamellipodia, that are similar to those used to drive cell migration, and thus may present adaptive variations of the same theme.

2.2.1. Adherens Junctions

Adherens junctions are adhesive structures found in all types of tissue. These cell-cell junctions are mediated by homophilic interaction between transmembrane proteins called cadherins together with two cytoplasmic components called catenins (Rudini and Dejana, 2008). Cell-cell junctions are enriched in actin filaments and the actin cytoskeleton plays an important role in both the
formation and maintenance of adherens junctions (Vasioukhin and Fuchs, 2001). Interestingly, when the dynamics of junctional actin were compared to the dynamics of junctional adhesive proteins by fluorescent recovery after photobleaching (FRAP) experiments, it was noticed that whereas the junctional adhesive proteins turn over quite slowly, actin is highly dynamic at the cell-cell junctions, suggesting that dynamic actin re-arrangements take place (Yamada et al., 2005). In epithelial cells, the small GTPase Rac1 has been strongly implicated in the formation of adherens junctions as blockage of Rac1 signaling disrupts the formation of these structures (Braga et al., 1997). The formation of membrane contacts that are necessary for the adherens junction assembly and maturation are driven by lamellipodial and filopodial structures (Vasioukhin et al., 2000; Ehrlich et al., 2002). For example, in keratinocytes, a zipper-like structure composed filopodia-like protrusions that are coated with E-cadherin precedes the formation of mature junction (Vasioukhin et al., 2000).

The actin cytoskeleton re-arrangements that assemble and maintain adherens junctions are driven by both Arp2/3 and formin mDia1 (Yamazaki et al., 2007; Carramusa et al., 2007). Arp2/3 activity is at least partially regulated by cortactin, which has a pronounced role in the formation and maintenance of cadherin-based epithelial adhesions possibly acting as a downstream effector of Src kinase (Helwani et al., 2004; Ren et al., 2009b). It is interesting to note, that the same set of molecules (e.g. the Rac pathway) that are utilized for the maintenance of cell-cell adhesions are also heavily engaged during cell migration/invasion. Loss of E-cadherin can result in disassembly of adherens junctions and epithelial to mesenchymal transition (EMT) (Klymkowsky and Savagner, 2009). Metastatic disease is thus a good example of how malfunction in upstream signaling pathways can lead to devastating changes from promoting vital (maintenance of adhesions) to destructive (promoting invasion) processes by utilizing exactly the same core set of actin-associated molecules.

2.2.2. Tunneling or membrane nanotubes (TNT’s) /cytonemes

Recent work has revealed the existence of thin tubular plasma membrane structures protruding from the plasma membrane and connecting cells over long distances called membrane nanotubes. Because these structures connect two cells, they can be distinguished from filopodia. Structurally, membrane nanotubes resemble filopodia as they both contain a core made of filamentous actin. However, whereas filopodia are highly dynamic, membrane nanotubes persist over long periods of time, from several minutes up to hours (Davis and Sowinski, 2008; Rustom et al., 2004b; Gerdes and Carvalho, 2008; Sherer and Mothes, 2008; Sowinski et al., 2008; Belting and Wittrup, 2008). Membrane nanotubes can have an open end, forming a tunnel which provides continuity between the cytoplasm’s of two cells. Alternatively, the membrane tube may be close-ended thereby forming a membrane bridge over two cells (Davis and Sowinski, 2008). Although the mechanisms that govern the formation and the maintenance of these structures are still relatively elusive, it seems that there are two distinct ways that can give rise to the formation of a membrane nanotubule: a filopodial precursor can fuse with the plasma membrane of another cell, or two cells that are in contact move away from each other leaving physical connection between them (Davis and Sowinski, 2008; Sherer and Mothes, 2008). Interestingly, the role of exocyst complex was recently identified as an important contributor for the formation of membrane nanotubes, which is not that surprising considering
that the exocyst has been previously linked to the formation of filopodia through the small GTPase RalA (Sugihara et al., 2002; Ohta et al., 1999). Also, the exocyst component Exo70 has been shown to interact with Arp2/3 (Zuo et al., 2006). These structures were first discovered in developing Drosophila embryo imaginal discs, where the existence of long tubular extensions was found to be associated with the transport of tissue morphogens over long distances (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999; Rustom et al., 2004a). More recently, these structures have been found in different mammalian cells such as T-cells, macrophages and dendritic cells. Membrane nanotubes can interconnect different types of cells thereby providing a transport route for intercellular cargo, such as vesicles and membrane proteins (Davis and Sowinski, 2008; Gerdes and Carvalho, 2008; Sherer and Mothes, 2008; Watkins and Salter, 2005; Gousset et al., 2009). As was the case in fruit fly, it seems that these structures are used for communication between mammalian cells. This is of great importance for the cells of the immune system, which are often spread around over long distances from each other. Accordingly, these structures have been shown to facilitate the exchange of cell receptors and signals between the interconnected cells (Davis and Sowinski, 2008). Also, it was recently shown in corneal explants that these structures can form between two MHC class II positive cells in vivo and the amount of nanotubes increased after tissue damage was introduced, indicating that they serve in cell-cell communication during inflammation (Chinnery et al., 2008).

Interestingly, HI-virus has adopted an interesting way of utilizing membrane tubes to aid its spread among T-cells. HI-virus can capture filopodia sent by another cell using a viral envelope glycoprotein. This will lead to the formation of a close-ended nanotube bridge between the two cells. After budding out of the host cell, HI-viruses can surf along the nanotube to the surface of the neighbouring cell using actin retrograde flow as a means of motility (Sherer and Mothes, 2008; Sowinski et al., 2008; Lehmann et al., 2005; Sherer et al., 2007). Interestingly, membrane nanotubes were recently also identified as a novel route for intercellular prion transmission (Gousset et al., 2009). Therefore, understanding the fundamentals of membrane nanotubes and filopodia will provide deeper understanding to many medically relevant issues. There are many important questions for the future research to resolve such as: how do filopodia find their target cells prior to forming tunnels? How are they anchored to the plasma membrane? What factors facilitate the possible membrane fusion events? How is the actin cytoskeleton regulated within these structures?

2.3. Endocytosis

Endocytosis describes processes, which cells utilize to internalize extracellular material. These are essential processes for the well being of cells, regulating e.g. receptor recycling, nutrient uptake and cell polarity. Endocytic processes are also intimately involved in the entry of different pathogens. There are various different endocytic pathways that are specified by a different set of effector molecules. Most of the endocytic routes involve the formation of plasma membrane invaginations through which the cargo is internalized (Doherty and McMahon, 2009). In addition to the pathways that operate through plasma membrane invaginations, two endocytotic mechanisms have been described that involve the formation of cell protrusions. These two processes are described in more detail below.

2.3.1. Phagocytosis

Phagocytosis describes an endocytotic event where cells engulf large extracellular
particles by forming protrusive structures that surround the particle to be internalized inside a sealed plasma membrane compartment. The pioneering work by Russian embryologist Ilya Metchnikoff over a century ago identified these structures as a part of host cell defence mechanisms (Metchnikoff’s work is highlighted in Tauber, 2003), and phagocytosis has since been established as a way to fight against invading bacteria, viruses and fungi. Phagocytosis is utilized by macrophages, neutrophils and dendritic cells and thereby contributes in both adaptive and initiate immunity. Besides microbes, phagocytosis is utilized to recognize apoptotic cells i.e. ‘altered self’ particles, which is important during tissue remodelling and embryogenesis (Flannagan et al., 2009; Stuart and Ezekowitz, 2008; Swanson, 2008).

The particle to be internalized is recognized by cell surface receptors directly, or indirectly via opsonins, to result in the clustering of the cell surface receptors and subsequent initiation of signaling cascade that induces actin remodelling through the activation of small GTPases Rac1 and Cdc42 (Caron and Hall, 1998). The actin-mediated re-arrangements seem to be executed by Arp2/3-dependent actin polymerization, although some formins have also been implicated to be important for phagocytosis (Brandt et al., 2007; May et al., 2000). Phosphoinositide signaling makes important contributions in the spatial-temporal regulation of phagosome formation. Sequential activities of PI(4,5)P_2 and PI(3,4,5)P_3 at the plasma membrane are important for the formation and sealing of phagosome, respectively (Fairn et al., 2009). Interestingly, one of the molecules that seem to respond to the switch in phosphoinositide signaling is myosinX, which drives the sealing of the phagosytotic cup (Cox et al., 2002). Also, the BAR domain protein amphiphysin-1 has been recently implicated to play a role in the phagocytotic process of testicular Sertoli cells by promoting actin polymerization at phagocytotic sites (Yamada et al., 2007).

### 2.3.2. Macropinocytosis

Macropinocytosis involves the deformation of plasma membrane in ruffles that can fuse back to the plasma membrane capturing extracellular material inside a membrane compartment. Similar event is also described as (circular) dorsal ruffling, which involves the cellular uptake of growth factor receptors through the formation of large membrane waves that capture extracellular material (Orth and McNiven, 2006). In order to capture extracellular material, ruffles need to adopt a cup-shaped morphology to form a cavity, which is subsequently constricted and fused to the plasma membrane from the distal ends. The newly formed vacuole undergoes membrane fission to result in an internalized membrane compartment that is not connected to the plasma membrane (Swanson, 2008; Mercer and Helenius, 2009). Macropinocytosis resembles phagocytosis in many ways and they share part of the downstream effectors that regulate actin reorganization during these events. It is known that Rac1 plays a significant role in the membrane ruffling by utilizing similar downstream effectors as described for the lamellipodia formation. However, in contrast to phagocytosis, macropinocytosis is not guided by specific cargo. Instead, macropinosomes can form spontaneously or in response to growth factor stimulation. Also, several viral and bacterial pathogens can induce macropinocytosis to promote their internalization (Swanson, 2008; Mercer and Helenius, 2009).

### 2.4. Bacteria-induced pedestals

Microbes have developed strategies that hijack host cell machinery to promote their infection and spread (see p. 26 and 33 for examples of virus-induced actin re-arrangements) Resolving the strategies
employed by microbes is beneficial from a medical point of view, but can also ideally teach us something about the mechanisms, which are utilized by the host cell for its ‘normal’ functions. Specific strains of *Escherichia coli* have developed strategies for colonizing the cells of the intestinal epithelia, which involve the formation of actin-rich cell surface protrusions called pedestals. Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli* are food-borne pathogens that induce loss of microvilli and diarrhoea upon colonization of the human intestine (Frankel and Phillips, 2008; Campellone and Leong, 2003). These bacteria make use of needle-like type III secretion system, which translocates intimin receptor Tir to integrate the host cell plasma membrane (Campellone and Leong, 2003). The mechanisms that lead to actin polymerization and pedestal formation in EHEC and EPEC differ somewhat from each other.

In EPEC, Tir can recruit adaptor protein Nck, which in turn recruits N-WASP to activate Arp2/3 complex mediated actin polymerization at the site of the bacterial attachment (Gruenheid et al., 2001). In contrast to EPEC, EHEC needs another secreted protein besides Tir for induction of pedestal formation. This protein is called EspFU, which translocates into the host cell cytoplasm. In the host cell of EHEC, Tir interacts with the I-BAR domain of IRTKS and/or IRSp53. These I-BAR proteins can in turn bind the C-terminal proline-rich motifs of EspFU via their SH3 domains (Vingadassalom et al., 2009; Weiss et al., 2009). EspFU is extremely powerful activator of actin polymerization by binding simultaneously five N-WASP molecules to promote pedestal formation (Cheng et al., 2008; Sallee et al., 2008). Recent *in vivo* study suggests that the corresponding mediator between Tir and EspFU is IRTKS and not IRSp53 (Crepin et al., 2009). In any case, the formation of this complex is crucial for the formation of the pedestals by EHEC, since the depletion of IRSp53/IRTKS abolishes pedestal formation. The discrepancy between the two primary findings linking I-BAR proteins to pedestal formation needs to be further investigated.

### 2.5. BAR proteins in membrane protrusions

Although the BAR proteins are better known for their role in the formation of plasma membrane invaginations such as in the formation of endocytotic vesicles, the above discussed literature indicates that many BAR domain containing proteins are also important contributors in the formation and/or maintenance of membrane protrusions. The over-expression of I-BAR domains leads to strong induction of membrane protrusions (Yamagishi et al. 2004) and thus it is not surprising that I-BAR proteins, such as IRSp53, have been found to play important roles in the formation of lamellipodia and filopodia (Suetsugu et al. 2006; Lim et al. 2008). There is also increasing evidence supporting the view that certain F-BAR proteins (now known as IF-BAR proteins) such as srGAP2, can bend the plasma membrane outwards to induce cell protrusions. Importantly, the protrusion formation activity of srGAP2 was found to be important for the proper development of the central nervous system (Guerrier et al. 2009). Surprisingly, some of the canonical BAR proteins are also seen in cell protrusions. For example, amphiphysin was found to localize to phagocytotic sites and membrane ruffles. It is important to note that there is also positive membrane curvature present at the sites of membrane protrusions, for example, at the neck of membrane ruffles or filopodia (Figure 1), which could partially explain why these proteins are localized to these sites.
AIMS OF THE STUDY

Prior to this thesis work, a family of distantly related proteins were identified (now known as I-BAR proteins), which were potent in inducing cell protrusions (Yamagishi et al. 2004). Some members of this novel protein family had already been characterized as regulators of actin dynamics in mammalian cells (Mattila et al. 2003, Woodings et al 2003, Miki et al. 2000, Krugmann et al. 2001), however, the molecular mechanisms how these proteins induce protrusions had remained poorly understood. Moreover, there were several uncharacterized members in this protein family with unknown function, and the role(s) of these proteins in animal physiology had not been investigated. Therefore, the aim of this study was to investigate the mechanisms and cellular/physiological roles of different I-BAR proteins by using a combination of biochemical, cell biological and genetic approaches. Specific aims were:

1. To characterize the mechanism(s) how I-BAR domains induce cell protrusions.
2. To reveal the expression pattern, actin- and membrane-binding mechanism, and the cell biological function of the novel I-BAR protein ABBA.
3. To analyse the phenotype of MIM knockout mice.
## METHODS

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Table 2. Experimental methods personally applied in this study. Roman numbers indicate the respective publications where they are described in detail.
RESULTS

3. Identification of a novel class of BAR proteins

Pioneering work by Yamagishi and colleagues identified a conserved protein domain in the N-terminus of five mammalian proteins, which was named IMD (IRSp53/MIM homology domain) hereafter referred as I-BAR domain. This work identified this conserved domain as the minimal region for the induction of protrusions by these proteins in mammalian cells. The proposed mechanism for the induction of cell protrusions by I-BAR domains was through bundling of actin filaments (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005).

Based on phylogenetic analysis, the mammalian I-BAR domain containing proteins can be sub-divided into two distinct branches, the MIM/ABBA branch and IRSp53/IRTKS branch (Yamagishi et al., 2004; Scita et al., 2008). Already prior to the identification of the I-BAR domain, two members of this protein family, MIM and IRSp53 had been linked in the formation of cell protrusions such as filopodia and lamellipodia (Mattila et al., 2003; Miki et al., 2000; Krugmann et al., 2001). Interestingly, when the structure of human IRSp53 I-BAR domain was solved, it was found to resemble the structures of the BAR domains, as it was composed of α-helical bundle that formed an antiparallel dimer.

3.1. The formation of cell protrusions by I-BAR domains is independent of small GTPase binding and does not involve actin bundling by I-BARs

Previously, it had been suggested that the I-BAR domain is a potent actin filament cross-linking module and that the formation of cell protrusions upon over-expression of I-BAR domains are a result of this activity (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005). When we repeated the low-speed actin co-sedimentation assays in physiological ionic conditions, we found only very weak actin bundling activity associated with the I-BAR domains in comparison with known actin bundling protein α-actinin (Fig. 5, III Fig. 4). When the salt concentration was decreased, the F-actin bundling activity was increased, suggesting that the I-BAR domain has a tendency to aggregate in low ionic strength. This salt-dependent aggregation of MIM I-BAR domain was confirmed by dynamic light scattering analysis (DLS), which demonstrated increased accumulation of I-BAR protein aggregates in low salt concentrations (I, Fig. 5). This suggested that the previously observed actin bundling activity of the I-BAR domains might be an artifact due to experimental conditions and therefore we wanted to visualize the exact localization of I-BAR domains in cells. Analysis in both U2OS and C6R cells revealed that I-BAR domains do not localize to F-actin bundles, but instead localize to the plasma membrane. Therefore, in filopodia, which are generally composed of a bundled F-actin core, I-BAR domain signal was found at the plasma membrane, but not in the F-actin shaft (I Fig. 5, III Fig. 4).

Interestingly, our sequence database searches revealed the existence of two alternatively spliced variants of MIM I-BAR domain. The shorter variant skips exon 7, whereas the longer MIM I-BAR variant includes exon 7 that encodes a four amino acid stretch located to the distal ends of the dimeric I-BAR domain (I Fig.S2). Both the shorter and longer splice variants bind and deform membranes in a similar manner, however, differences were found in their ability to interact with the small GTPase Rac1. The shorter variant was found to efficiently bind to Rac1 in
Results

GST-pull down assays whereas no binding between Rac1 and the longer variant was detected. The binding between Rac1 and the two MIM splice variants was further investigated by surface plasmon resonance analysis (SPR), which demonstrated a clear difference in the binding of the different variants to Rac1. Consistent with the results from the pull-down assays, the shorter I-BAR variant was found to bind Rac1 in a concentration-dependent manner, whereas the binding of the longer variant was poorly detectable and was found to be only 10-15% that of the shorter variant equilibrium binding level values (I, Fig. 6). No interaction between the I-BAR domains with the small GTPase Cdc42 was found in these assays (III Fig. S3, and data not shown). The discovery of Rac-binding deficient I-BAR domain enabled us to investigate the role of Rac1 binding in the formation of I-BAR domain mediated cell protrusions. Thus we quantified the amount of filopodia-like protrusions in U2OS cells over-expressing either the short or long MIM I-BAR variants, but found no apparent differences, suggesting that Rac1 binding has no obvious role in the formation of membrane protrusions by I-BAR domains.

3.2. Inverse-BAR domains bind phosphoinositide-rich membranes and bend them in the opposite direction as canonical BAR domains

Given the pronounced role of BAR domains in membrane dynamics, we wanted to test if the I-BAR domain is a membrane binding module. We tested the binding of I-BAR domains to membranes by native gel electrophoresis and vesicle co-sedimentation assays and found that I-BAR domains bind to lipids, favoring negatively charged phosphoinositides PI(4,5)P2 and PI(3,4,5)P3. Extensive alanine scan mutagenesis revealed that the binding site is located at the distal ends of the I-BAR dimer and involved a patch of positively charged residues. Interestingly, these residues were located at the convex face of the I-BAR domain, whereas in BAR and F-BAR domains the lipid-binding surface had been mapped to the concave domain surface (Peter et al., 2004; Henne et al., 2007; Shimada et al., 2007). To visualize the effects of I-BAR domains on the morphology of the lipid vesicles, we incubated synthetic membrane vesicles with different I-BAR domains and visualized them with transmission electron microscopy (TEM). Surprisingly, we found strong membrane deformation activity associated with I-BAR domains. The membrane vesicles were deformed into tubular structures in a PI(4,5)P2 concentration-dependent manner (I, Fig. 1; II Fig. 3). The diameters of I-BAR induced membrane tubules displayed relatively consistent diameters although differences in the mean diameter of tubules induced by different I-BAR domains were detected (II, Fig. 3).

The BAR and F-BAR domains bind and deform membranes via their concave membrane binding surface. If these domains are exogenously expressed in cells, they induce the formation of plasma membrane invaginations. Consistent with these findings, cryo transmission electron microscopy (cryo-EM) analysis has revealed that F-BAR domains bind to the outer leaflet of the membrane tubules (Frost et al., 2008). In sharp contrast to BAR and F-BAR proteins that induce cell invaginations, I-BAR domains promote the formation of cell protrusions when expressed in cells. To comprehensively analyze the directionality of I-BAR domain induced membrane deformation in vitro, we utilized a setup composed of fluorescently labeled giant unilamellar vesicles (GUV) and purified protein components. This analysis enabled us to follow single vesicles live and to visualize the effects of BAR proteins on the vesicle morphology upon addition of the protein
The binding of I-BAR domains to the membranes involves clustering of PI(4,5)P_2

Interestingly, when we imaged GUVs that were labeled with two fluorescent probes: NBD-labeled phosphatidycholine and bodipy-TMR-labeled PI(4,5)P_2, we noticed that the addition of different I-BAR domains and the N-BAR domain of amphiphysin resulted in clear accumulation of PI(4,5)P_2, but not PC, on surface of GUVs. These clusters appeared to be relatively stable and were often associated with the formation of membrane tubules (II, Fig. 2). To quantify this effect, we applied a fluorometric PI(4,5)P_2-clustering assay that is based on self-quenching of bodipy-TMR-label. We found that all I-BAR domains clustered PI(4,5)P_2 efficiently. Also the N-BAR domain of amphiphysin clustered PI(4,5)P_2 although not as efficiently as I-BAR domains. By testing various mutants, we were able to demonstrate that the clustering of PI(4,5)P_2 was facilitated through electrostatic interactions between the positively charged residues at the distal end of the I-BAR. Importantly, I-BAR domains were unable to cluster phosphatidylserine, which is another negatively charged lipid, indicating that the clustering is relatively specific towards PI(4,5)P_2 (II, Fig. 2).

Together, these results suggest that the I-BAR and other BAR domains are capable of inducing phosphoinositide enriched sites at the plasma membrane.
S4). These results suggested that there are differences in the mechanism of membrane interaction between different I-BAR domains.

To test if this is the case, we compromised electrostatic interactions by increasing salt concentration in the reaction buffer (up to 400 mM NaCl) and measured the binding of the I-BAR domains of MIM and IRSp53 to vesicles in a co-sedimentation assay. As expected, we found that the binding of IRSp53 I-BAR to the membranes was severely compromised whereas, surprisingly, MIM was still efficiently bound to membranes (II, Fig. 4). This result indicated that MIM (and ABBA) might harbor additional membrane binding motifs besides electrostatic interactions. To investigate if I-BARs insert into the membrane bilayer, we studied the steady-state fluorescence anisotropy of membrane probe DPH, which locates to the hydrophobic core of membrane bilayer and can be applied to monitor changes in the trans-gauche isomerization of phospholipid acyl chain region. Interestingly, binding of both MIM and ABBA I-BAR domains to membrane induced significant increase in the DPH-anisotropy, indicating that these proteins insert into the membrane bilayer. Also, the I-BAR domain of C.elegans increased the DPH anisotropy although the increase was not as prominent as in the case of MIM and ABBA. In contrast, the I-BAR domains of IRTKS and IRSp53 did not increase DPH anisotropy indicating that they do not insert to the membrane bilayer. The crystal structure of mouse MIM I-BAR domain (Lee et al., 2007) hinted that the potential insertion motif might lie in the N-terminal helix, which displayed amphipathic properties with polar residues distributed on one side of the helix flanked by non-polar residues (II, Fig S4). The N-terminal part has also been shown to be responsible for membrane insertion in N-BAR domains (Gallop et al., 2006; Masuda et al., 2006). To test this, we made an N-terminally truncated MIM I-BAR mutant where the first 11 amino acids are removed (MIMΔN) and analyzed its membrane interaction with DPH anisotropy. Surprisingly, we found that MIMΔN did not insert into the membrane. When the membrane deformation activity of this mutant was analyzed with TEM, it was noticed that although the mutant tubulated membranes efficiently, the width of the tubules was significantly reduced when compared with wild-type MIM I-BAR and was in the same range as the width of tubules induced by IRSp53 I-BAR, which does not insert into the membrane bilayer (II, Fig.5 ). Together, these data revealed a clear difference in the mechanism of membrane interaction between MIM and IRSp53 I-BAR domain sub-branches, and further propose that an insertion of a N-terminal helix to the membrane by MIM and ABBA I-BAR domains increases the diameter of membrane tubules induced by these domains.

3.5. I-BAR domains induce cell-protrusions that are dependent on intact membrane-binding interface of the domain and the actin cytoskeleton

Previous studies had demonstrated that over-expression of I-BAR domains in cells results in a massive formation of cell protrusions containing filamentous actin (Yamagishi et al., 2004; Millard et al., 2005). Our live cell imaging experiments demonstrated that the cell protrusions induced by I-BAR domains were highly dynamic, displaying rapid elongation and retraction rates. To analyze the role of lipid binding of I-BAR domain to the formation of filopodia-like protrusions in cells, we compared the filopodia formation rates between wild-type MIM and mutants that display compromised lipid-binding activities. When compared with the wild type MIM, the mutant which lacked lipid-
binding and membrane deformation activities (K138,139,146,149,150A) was unable to induce filopodia-like protrusions. However these mutations also overlapped with the proposed F-actin-binding interface of the I-BAR domain. Therefore, we analyzed the formation of filopodia-like protrusions upon exogenous expression of MIM I-BAR mutant that displayed a moderate defect in lipid-binding but not in F-actin-binding activities. Importantly, this mutant displayed significantly reduced the formation of filopodia-like protrusions, demonstrating that the lipid-binding by the I-BAR domain is critical for its filopodia forming activity in cells. To test the role of the membrane insertion in the formation of cell protrusions, we compared the filopodia formation activities between wild type MIM I-BAR, MIMΔN and IRSp53 I-BAR domain. This analysis revealed that when expressed in cells, the wild-type MIM I-BAR domain was significantly more efficient in inducing filopodia-like membrane protrusions in comparison with MIMΔN or IRSp53 I-BAR domains, demonstrating that the N-terminal membrane insertion enhances the formation of I-BAR-mediated filopodia-like membrane protrusions in cells (II, Fig.5).

To test the role of the actin cytoskeleton for the formation of these protrusions, we used a drug called latrunculin A, which binds and sequesters actin monomers thereby preventing actin polymerization. This analysis revealed that intact actin cytoskeleton is necessary for the elongation of I-BAR induced protrusions. It is important to note that disruption of the actin cytoskeleton did not lead to a total collapse of these cell protrusions (I Video S, III Fig. S4). These results indicate that polymerization of the actin cytoskeleton provides force for the filopodia elongation and the submembranous I-BAR domains might be important for the initiation and stabilization of membrane curvature (Figure 9). Some studies have reported that at least part of the I-BAR domains induced plasma membrane protrusions that lack F-actin completely as analyzed by immunofluorescence microscopy (Suetsugu et al., 2006; Lim et al., 2008). A recent study focused on this controversial aspect and found that the actin cytoskeleton is required for the elongation and long-term maintenance of I-BAR domain induced membrane protrusions. However, they also found that I-BAR mediated membrane deformation may precede actin assembly during filopodia formation (Yang et al., 2009). Interestingly, platinum replica EM analysis performed in this study provided information about the organization of actin filaments in these protrusions. It seems that in I-BAR domain induced protrusions the actin filaments are less-well organized than in typical filopodia, where filaments are typically aligned in a tight parallel bundle (Yang et al., 2009). Collectively, these data suggest a model where initial membrane protrusion is generated through I-BAR domain mediated membrane deformation and this membrane bending is sufficient to cluster actin filament barbed ends from underlying branched actin network into weakly bundled arrays (Liu et al., 2008). Subsequently, the elongation and the growth of these filaments provide force for protrusion elongation (Figure 8). However, the role of established regulators of actin dynamics in such protrusions was remained unclear.

3.6. I-BAR domain induced cell protrusions contain filopodial markers

In order to test if the filopodia-like protrusions induced by exogenous expression of I-BAR proteins can be classified as functional filopodia, we analyzed the presence of established filopodia markers in membrane protrusions induced by GFP-tagged full-length MIM and IRSp53 or their respective I-BAR domains in U2OS, Hela and COS-7
cells. First, cells expressing the above indicated constructs were stained with fluorescently-labelled myosin-X antibody. Myosin-X is a motor protein, which is often found concentrated in the tips of filopodia and can also move back and forward along filopodia shafts (Berg and Cheney, 2002). Interestingly, in many cases myosin-X was seen in both full-length MIM and IRSp53 as well as I-BAR domain induced filopodial tips and shafts. Additionally, when cells were co-transfected with MIM-I-BAR domain and GFP-tagged myosin-X construct, we found that myosin-X displayed movement along I-BAR induced filopodia-like protrusions (II, Video 2). Moreover, many other filopodial markers such as fascin and VASP were found localized in MIM I-BAR induced filopodia-like protrusions. In conclusion, these experiments provide evidence that the protrusive structures, which are formed upon over-expression of various I-BAR constructs and full-length I-BAR proteins display similar organization and characteristics as filopodia (II, Fig. S5); (Figure 8).

3.7. Mammalian I-BAR domains display dynamic interaction with membranes whereas the nematode homolog forms more stable and rigid structures

To test if there are differences in the dynamics of membrane interaction between different I-BAR domains, we transfected U2OS cells with different GFP-tagged I-BAR constructs and measured their exchange rates in filopodia by fluorescence-recovery-after-photobleaching (FRAP). Surprisingly, we found no significant differences in the exchange rates between MIM and IRSp53 I-BAR domains, which displayed half-time recovery rates of ~8 and ~5 seconds, respectively. Also, MIMΔN I-BAR displayed similar recovery rates as the wild type MIM I-BAR (unpublished data).

Figure 8. Hypothetical model for I-BAR domain induced protrusion formation. I-BAR domain can sense/generate plasma membrane curvature, which attracts more I-BAR domains through co-operative binding to result in the deformation of the plasma membrane into a short tubular protrusion and simultaneous clustering of PI(4,5)P₂ at the membrane. Deformation of plasma membrane clusters underlying actin filament barbed ends from branched network into straight actin filament arrays, which are loosely packed together by the surrounding plasma membrane. Actin filament polymerization against the plasma membrane generates the necessary force for the protrusion expansion. Actin dynamics are regulated by different actin-binding proteins that may be attracted and activated by local increase in PI(4,5)P₂ concentration.
These data suggested that the membrane insertion does not significantly alter the dynamics of membrane interactions of I-BAR domains. In striking contrast, the C. elegans I-BAR domain displayed very slow recovery indicating that this domain might make stable intermolecular contacts between adjacent domains within filopodia that display regular diameter (II, Fig. 6). This is further supported by clear segregation of C. elegans I-BAR domain from MIM and IRSp53 I-BAR domains in cells, and the more rigid nature of the membrane protrusions induced this domain both in cells and in vitro (based on cryo-EM analysis). These data suggest that whereas the mammalian I-BAR domains exchange rapidly in filopodia, the nematode I-BAR domain oligomerizes into relatively stable lattices, similarly as has been previously been shown for some F-BAR domains (Frost et al., 2008).

4. Cellular and physiological roles of I-BAR proteins in mammals

In order to elucidate the cellular and physiological role of I-BAR domain containing proteins in mammals, we examined the role of MIM/ABBA proteins in cellular systems by using exogenous expression constructs as well as RNAi approaches. Furthermore, much effort has been placed in analyzing mice where the expression of MIM gene is disrupted. Together, these approaches have made it possible to investigate the importance of different biochemical activities of these proteins in the context of the full-length protein in different cell-based systems as well as appreciate the importance of these proteins in the complicated context of animal physiology.

4.1. ABBA is highly expressed in glial cells during development

ABBA is the closest homologue of MIM and these proteins display ~58% identity to each other at the amino acid sequence level. MIM and ABBA proteins are highly conserved in the regions corresponding to the I-BAR and WH2 domains whereas the region between these N and C-terminal ends displays substantial differences, suggesting that these proteins might have at least partially different roles in cells. In order to study the expression of ABBA during mouse development and in adult mouse tissues, we conducted an exhaustive RNA in situ hybridization screen to visualize the expression pattern of ABBA gene and compared it to the expression of MIM. Intriguingly, this analysis revealed that ABBA and MIM mRNAs are differentially expressed during development. For example, at E12.5 MIM is expressed in a sub-population of neurons located at the ventral portion of the neural tube (III, Fig. 1) as well as in the developing heart and myoblasts (Mattila et al., 2003). In contrast, the expression of ABBA at E12.5 was strong in the developing central nervous system, and was found especially enriched in the transient glial structure called floor plate, and in the marginal zone of the developing cortex (III, Fig. 1). This suggested that ABBA is expressed in glial structures and the notion of ABBA mRNA in the outer edge of the marginal zone suggested that ABBA mRNA might be transported from the ventricular zone, where the radial glial cell bodies lie, to the marginal zone, which is situated in the outer margins of the cortex. This is not that surprising since in recent years, sub-cellular mRNA targeting has emerged as an important mechanism in maintaining many different cell biological aspects including cell polarity (Holt and Bullock, 2009). In addition to CNS expression, ABBA was expressed in the developing bones at E12.5. At E14.5-18.5 the expression of ABBA was predominant in the midline glial structures and on the pial surface lining the brain parenchyma. In adult mice, our Northern blot analysis indicated that the strongest expression of ABBA is found
Results

in the brain with moderate expression in different tissues including the testis, liver, kidney and skeletal muscles (Fig. 1). The RNA in situ hybridizations conducted to sections representing different mouse adult mouse tissues confirmed this and provided more specific information on the spatial expression pattern within tissues. For example, in adult mouse testis, ABBA mRNA was specifically expressed in the interstitial Leydig cells. In adult brain, ABBA was predominantly found in the molecular layer of the cerebellum and was also expressed moderately throughout the brain parenchyma (Fig. 1).

To confirm that the results obtained from the RNA in situ hybridizations are also valid at protein level, we generated a polyclonal antibody in rabbits against the ABBA protein fragment composed of amino acids 274-683. This antibody did not cross-react with other I-BAR proteins and was thus used to perform immunohistochemical analysis to detect ABBA protein in the developing brain. This analysis correlated perfectly with the results obtained from the in situ hybridizations. Furthermore, double labeling tissues sections with ABBA antibody together with neuronal (Tuji) and glial markers (R2 and GFAP) demonstrated that ABBA is expressed in glial but not in neuronal cells during the development of the CNS (Fig. 2 and data not shown). In adult cerebellum, ABBA was found in the Bergmann glial cells as well as in the extensions of the Purkinje cells.

4.2. ABBA is localized to the interface between the plasma membrane and the cortical actin cytoskeleton through its I-BAR domain

To examine the sub-cellular localization of ABBA we used C6R cells, which display characteristics of radial glial cells (Friedlander et al., 1998). Unlike primary radial glia, these cells can be transfected and are easy to culture in vitro. Staining these cells with ABBA antibody revealed that ABBA localizes to the plasma membrane in front of the cortical actin cytoskeleton where the barbed ends of actin filaments are situated (Fig. 3). In order to analyze what region in ABBA was responsible for its localization to the cell cortex, we expressed various mutant versions of GFP-tagged ABBA in C6R cells and analyzed their sub-cellular localization. This analysis revealed that mutations disrupting the membrane-binding of ABBA I-BAR domain abolish the localization of full-length ABBA form the cell cortex, whereas the inactivation of WH2 domain or deletion of the central polyproline regions had no obvious effect on the sub-cellular localization of ABBA (Fig. S4).

4.3. ABBA binds ATP-actin monomers with a high affinity

We also investigated the biochemical activities of the C-terminal WH2 domain of ABBA in actin-binding. Importantly, a fluorometric assay with NBD-labeled actin demonstrated that ABBA WH2 domain binds “polymerization competent” ATP-bound actin monomers with a high affinity (181 nM) and ADP actin monomers with a significantly lower affinity (676 nM). When the canonical LRR-motif in the WH2 of ABBA was mutated into alanines, the binding to actin monomers was abolished (Fig. 4). These results suggest that in cells, ABBA functions at regions of rapid actin dynamics.

4.4. ABBA regulates the extension of glial cells through its membrane binding/deforming activity

To investigate the biological role of ABBA in radial glial cells, we used siRNA-oligos targeted against ABBA to decrease its expression levels. The RNAi-mediated knockdown revealed no obvious changes
in the C6R cell morphology or in the formation of filopodia. Therefore we decided to challenge the knockdown cells to assess the dynamics of their cortical actin cytoskeleton. We reasoned that because ABBA is expressed in the end processes of radial glial cells and localized to the sites of rapid actin dynamics, that it might be involved in the extension process of these highly polar cells. Thus, we monitored the growth of freshly plated C6R extensions in both laminin and collagen coated substrata by using automated cell culturing platform (Cell-IQ). During the 12 hour monitoring period, cells were automatically imaged at specific intervals and length of the extensions was measured over time. This analysis revealed that ABBA knockdown cells had significant disturbances in their process outgrowth (III, Fig. 6). To confirm that the effect seen was due to decreased ABBA expression, we transfected ABBA knockdown cells simultaneously with GFP-ABBA construct that had a silent mutation in the siRNA target site making it resistant to the RNAi oligonucleotides. Importantly, we found that this construct could rescue the defect seen upon ABBA knockdown. We also tried to rescue the phenotype with various ABBA mutants and found that intact I-BAR domain was essential for the role of ABBA in C6R cell extension whereas the WH2 mutant could still efficiently rescue the cell extension defect (III, Fig. 6).

C6R cell extension is driven by lamellipodial actin dynamics at the polar edges of the growing cell. Therefore, we wanted to analyze lamellipodial dynamics of these cells. To this end, we took videos from the leading edges of freshly plated C6R cells and subjected these videos to kymograph analysis, where lamellipodia extension and retraction velocities and frequencies can be analyzed (Hinz et al., 1999). Importantly, this analysis revealed a significant decrease in the ruffling frequency and velocity of individual protrusions in comparison with cells transfected with control oligos (III, Fig.7). The exact mechanism how ABBA contributes to lamellipodial dynamics remains uncertain, but it is possible that sensing membrane curvature by I-BAR domain proteins might be important for recruiting actin polymerization machinery to the right spot at the plasma membrane and this might involve active membrane bending by I-BAR domain to further promote protrusion formation, possibly enchancing actin polymerization (see Discussion, Figure 10). Interestingly, we also found that ABBA binds to the small GTPase Rac1 via its I-BAR domain. This interaction further imposes the role of ABBA in lamellipodial dynamics although the importance of this interaction remains to be investigated.

4.5. MIM is dispensable for mouse development

In order to understand the biological role of I-BAR proteins in the context of animals, it is necessary to generate transgenic animals that lack genes encoding these proteins. We generated a mouse line where we inactivated MIM gene by inserting a Neomycin gene in the translation initiation codon-containing exon 1.

Previous studies had also implicated MIM as a sonic hedgehog (Shh) responsive gene that interacts with Gli1/2 transcription factors and enhances their transcription (Callahan et al., 2004). Shh is a morphogen that drives many patterning events during development and disturbances in Shh-signaling lead to severe developmental abnormalities (Varjosalo and Taipale, 2008). Therefore, we were surprised to find that our MIM knockout mice developed normally into adulthood and displayed no gross abnormalities (IV, Fig.1). We thus wanted to re-assess the role of MIM in Shh-pathway. Shh is a secreted molecule, which binds in its target cells to the transmembrane receptor patched (Ptc). Ptc is a repressor
of another membrane protein Smoothened (Smo). Binding of Shh to Ptc releases Smo to activate downstream signaling, which ultimately leads to the activation of Gli1/2 transcription (Varjosalo and Taipale, 2008). To study the role of MIM in Shh-signaling pathway in animals, we utilized Ptc-/- mice where Shh signaling is constitutively active. Ptc-/- mice die at E9-10.5 due to abnormal development of the heart. We reasoned that if MIM is important for Gli1/2 transcription, loss of MIM should yield in reduced Shh-mediated transcription and should therefore at least partially rescue the lethal phenotype of Ptc-/- mice (Bai et al., 2002). To test this, we crossed our MIM knockout mice with Ptc mice and analysed the phenotype of the double targeted mice. Surprisingly, we did not observe any rescue nor did we see any differences in the expression of the marker gene β-galactosidase (β-gal), which was under the Ptc promoter and made possible to analyze the relative Ptc expression in different MIM genotypic backgrounds (II Fig.S3). These data suggested that MIM does not modulate Gli1/2 transcription during animal development. These data was further corroborated by our cell-based Shh-reporter assays where the role of MIM in Gli-transcription was evaluated. Importantly, no effect was detected by either MIM or ABBA in Gli transcription in this assay. Moreover, MIM failed to co-immunoprecipitated with Gli1 or Gli2 (II Fig.S3). Collectively, these data show that MIM does not regulate Gli-transcription in animals or in cultured cells, as previously reported.

4.6. MIM deficiency leads to compromised renal functions and consequent bone abnormalities

Since MIM is highly expressed in the adult kidneys (IV, Fig. S4); (Mattila et al., 2003), we decided to analyze the renal functions of MIM knockout mice together with their control wild type littermates by placing these mice in metabolic cages. This allowed us to monitor their daily water intake as well as urine output levels and to do subsequent urine analysis. Interestingly, these analyses revealed a significant increase in both daily water intake and urine output levels in the knockout animals. In addition, the knockout mice displayed urinary wasting of electrolytes such as calcium, magnesium, potassium and sodium (IV, Fig. 2). Collectively, these data suggested that MIM knockout mice suffered from reduced tubular water and electrolyte intake.

Malfunction of the renal system is commonly associated with alterations in bone metabolism in human patients suffering from chronic kidney disease (Magnusson et al., 2001). Clinical chemistry analysis revealed a significantly increased level of plasma alkaline phosphatase (ALP) in the MIM null mice. Plasma ALP is an established marker for bone metabolism in patients suffering from kidney disease (Magnusson et al., 2001). This led us to investigate the bones of MIM mice. Interestingly, bone densitometry analysis using dual-energy X-ray absorptiometry (DXA) unveiled a significant decrease in both bone mineral content and bone content in MIM knockout mice when compared with wild type littermates (IV, Fig. 3). As no genotype-specific differences were found in the bodyweights or bone lengths of these mice, we believe that the differences detected in the bones represent a secondary effect due to the kidney defect in the MIM knockout mice.

4.7. MIM deficient mice display morphological alterations in the kidney

We did not detect any histological alterations in the kidneys of young (<2 month) MIM knockout mice. However, as the mice aged they started to appear sick. It is important to note that there was a
large variability (from 6 to 18 months) in the onset of the first symptoms. The sick mice were analyzed histopathologically and this revealed dramatic changes in the kidney morphology (IV, Fig. 5). The first histological signs were found in the tubular structures, which had a dilated appearance. As the disease progressed, there was apparent infiltration of lymphocytes as well as glomerulosclerosis. Finally, massive fibrosis and total degeneration of glomeruli was detected.

To get more comprehensive view on what is the primary cause of this disease, we utilized TEM to analyze the ultrastructure of kidneys of three MIM knockouts that displayed urinary concentration defect but did not appear sick, together with their three healthy littermate controls. This analysis revealed apparent atrophic changes in the tubular epithelium of MIM knockout mice. The lateral intercellular spaces between adjacent proximal tubule cells were found dilated whereas these changes were not observed in the wild type littermate controls. Furthermore, this analysis revealed that the basal membranes in the MIM knockout mice were thickened (IV, Fig.). Taken together the histological data and the results obtained from the metabolic cage experiment, it was apparent that loss of MIM leads to compromised integrity of kidney epithelium, altered urine handling and consequent bone abnormalities possibly due to altered calcium homeostasis.

4.8. MIM displays dynamic localization to adheres junctions where it promotes F-actin assembly

In order to study the molecular mechanism behind these observed pathological changes in MIM knockout mice, we took advantage of MDCK (Madin-Darby Canine Kidney Cells) cells that are easily transfected and can be polarized to adopt characteristic epithelial morphology in culture. First, we analyzed the localization of MIM in these cells. Since no antibodies against MIM were available that would work on immunocytochemistry, we transected these cells with a MIM GFP-construct. Interestingly, MIM co-localized with adherens junction marker E-cadherin to intercellular contacts. Next, we wanted to analyze what region in MIM protein is responsible its localization to cell-cell junctions. For this, we transfected MDCK cells with MIM-GFP construct where the I-BAR domain was inactivated by point mutations or where the C-terminal WH2 domain had been deleted. This analysis revealed that the I-BAR domain was necessary for the proper localization of MIM to cell-cell junctions whereas the WH2 domain was dispensable for the localization of MIM to these sites.

FRAP analyses have revealed that the adherens junction adhesion molecules E-cadherin and β-catenin display much slower dynamics in cell-cell contacts when compared with actin at the cell-cell junctions (Yamada et al., 2005). When we analyzed the dynamics of MIM in intercellular adhesions by using FRAP, we found that MIM was dynamically exchanged at these sites and displayed similar recovery rates as has been previously reported for actin and many actin-binding proteins (IV fig.6 and (Yamada et al., 2005).

Interestingly, we also noticed that exogenous expression of MIM resulted in brighter F-actin staining at cell-cell contacts when compared with neighboring non-trasfected cells. When we quantified this effect, we found that MIM increased the mean F-actin staining intensity at intercellular junctions by ~17 %. When we analyzed the F-actin staining intensity in cells transfected with MIM I-BAR mutant that did not localize correctly, this effect was severely decreased. Importantly, when we carried out the same analysis for the MIM construct lacking the C-terminal WH2 domain, we observed a significant decrease compared with wild-type MIM,
indicating that WH2 domain plays an important role in the F-actin assembly seen upon exogenous MIM expression in MDCK cells. Together, our cell biological assays suggest that MIM might be important regulator of actin polymerization at the cell-cell contacts. For this, both intact I-BAR and WH2 domains seem to be critical. MIM-mediated actin assembly most likely also involves interactions with proteins, which are directly regulating the actin polymerization machinery. A good candidate in mediating the MIM induced actin assembly at these sites is cortactin (Lin et al., 2005), which has previously been shown to be important for the maintenance of adherens junctions of epithelial cells (Helwani et al., 2004; Ren et al., 2009a).
DISCUSSION

A vast number of cellular processes occurring at the plasma membrane involve bending of the membrane either outwards or inwards. The most famous examples of these are endocytic and migratory events. The force that generates these membrane deformation events is obtained from cytoskeletal and/or membrane deforming protein scaffolds. This study concentrated on investigating a novel class of membrane deforming proteins called I-BAR proteins, which intriguingly combine these two different mechanisms by intimately linking direct membrane deformation to the regulation of the actin cytoskeleton, which can act synergistically to drive the formation of cell protrusions.

The study by Yamagishi et al. demonstrated that the N-terminal part of MIM/IRSp53 molecules corresponding to the I-BAR-domain is the minimal region in these proteins to induce the formation of F-actin rich plasma membrane protrusions in cells. Mechanistically, it was proposed that I-BAR domains function by cross-linking actin filaments, which was demonstrated by experiments showing actin-filament bundling activity for this domain in vitro (Yamagishi et al., 2004; Millard et al., 2005; Gonzalez-Quevedo et al., 2005; Millard et al., 2007). However, later on, the actin cross-linking activity of the I-BAR domain has been subjected under dispute, since we and others have found it to be very weak under physiological ionic conditions when compared with established actin-bundling proteins (I Fig.5; Lee et al., 2007). The weak bundling activity found in in vitro experiments was further corroborated by the sub-cellular localization of I-BAR domain to the plasma membrane instead of actin bundles (I Fig.4).

Intriguingly, the crystal structures of IRSp53 and MIM I-BAR-domains revealed clear homology to the structure of BAR domains. However, whereas the BAR domains are curved, the I-BAR is only gently curved α-helical antiparallel dimer (Millard et al., 2005; Lee et al., 2007). Inspired by the structural homology to the BAR domains, we and others studied whether there is also functional homology between these protein modules. Indeed, these attempts revealed that I-BAR domains bind to phosphoinositide-rich membranes with high affinity and are capable of deforming them into tubular structures (I Fig 1; Suetsugu et al., 2006). The mechanism by which I-BAR proteins execute their membrane bending functions involves strong electrostatic interactions between the positively charged residues located at the distal ends of the I-BAR dimer and the negatively charged phosphoinositide headgroups (I, Fig. 2B). Interestingly, we found that the membrane binding of I-BAR domains also involves clustering of PI(4,5)P₂, which is the most abundant phosphorylated derivative of phosphatidylinositol in mammalian cells. Although the concentration of PI(4,5)P₂ is considered to be relatively stable (between 0.3 – 30% depending on the cell type), spatial alterations in the PI(4,5)P₂-concentration can be achieved through the action of specific PI kinases and/or phosphatases and, importantly, spatial concentration changes of phosphoinositides at the plasma membrane are commonly described at the sites of rapid actin dynamics (Saarikangas et al., 2010). In the future it will be important to try to elucidate out the exact mechanism of the PI(4,5)P₂ clustering by I-BAR domains, for example, by comparing the dynamics of I-BAR array versus PI(4,5)P₂ in these clusters as well as to try to understand the importance of this phenomenon in the context of cells. The PI(4,5)P₂ clusters may have an important role in acting as signaling platforms, which may provide away to promote actin dynamics in a spatially
controlled manner (Saarikangas et al., 2010). For example, it has been shown that the activity of the actin nucleation promoting factor N-WASP is regulated by an increase in the PI(4,5)P_2 density. When a certain threshold concentration of local PI(4,5)P_2 is achieved, the autoinhibition of N-WASP is released and it can activate Arp2/3-mediated actin polymerization (Papayannopoulos et al., 2005). This is especially intriguing considering that N-WASP is a binding partner of many BAR, F-BAR and I-BAR proteins (Kovacs et al., 2006; Lim et al., 2008; Tsujita et al., 2006). It was also recently suggested that during the formation endocytic membrane invaginations, the phosphoinositide clustering activity of BAR domain proteins might function to protect PI(4,5)P_2 from phosphatase activity, leaving the endocytotic bud-tubule interface vulnerable for the phosphatase activity. This would presumably lead to interfacial forces that squeeze the bud-tubule boundary, eventually resulting in scission of the vesicle and disassembly of coat proteins (Liu et al., 2009; Liu et al. 2010).

Importantly, a subset of I-BAR domains harbors an membrane insertion motif in their N-terminal region, which serves to enhance the membrane binding and deformation activities. It also contributes to the degree of membrane curvature being generated by acting as a wedge that induces bilayer asymmetry that should, in principle, give rise to positive membrane curvature. This is an interesting controversy, since all the I-BAR domains induce negative membrane curvature due to their strong electrostatic interactions with the membrane phospholipids. Hence, the question is raised: why do some I-BAR domains insert into the bilayer? In N-BAR domains, the insertion of amphipathic helices into the bilayer is crucial for their membrane deformation activity (Gallop et al. 2005; Masuda et al. 2005). However, this was not the case with I-BAR domains, as they could still deform membranes in the absence of inserting motifs, and thus the cellular role of membrane insertion in I-BAR proteins needs to be further investigated. The insertion might play a role in sensing positive membrane curvature, although the reason for a protein that induces negative membrane curvature (cell protrusion) to sense positive membrane curvature (cell invagination) is unknown. Based on the recent results by the Stamou group (Bhatia et al., 2009b; Bhatia et al., 2009a), all BAR, F-BAR and I-BAR domains sense positive membrane curvature. The initial membrane curvature might be derived from, for example, a local increase in PI(4,5)P_2 density. This should, in principle, generate positive membrane curvature due to local plasma membrane asymmetry caused by the large size of PI(4,5)P_2 headgroups at the inner leaflet. Initial inward bending of the plasma membrane can also be aided by clathrin adaptor proteins such as epsins that use a membrane insertion mechanism to drive membrane bending (Ford et al., 2002). If indeed all BAR domains use their membrane inserting motifs to sense positive membrane curvature, it is tempting to speculate that under certain circumstances there might be a constant tug of war between BAR-proteins that promote invaginations and BAR proteins that promote protrusions. Along these lines, a recent study suggested that the I-BAR protein MIM functions to inhibit endocytosis by competing with the N-BAR domain containing pro-endocytic protein endophilin for cortactin and CD2AP-binding (Quinones et al. 2010). This study showed that inactivation of MIM/ABBA homologue in Drosophila melanogaster results in a border cell migration defect. The authors concluded that MIM functions to inhibit endocytosis in a specific context to maintain more persistent guidance cue signaling by decreasing the endocytosis of EGFR and PDGF (Quinones et al. 2010). Based on these results, one could make an experimentally feasible hypothesis.
that MIM and endophilin, through their amphipathic helices, sense similar sites at the plasma membrane that display positive membrane curvature. In any case, further work needs to be done to test if different BAR proteins indeed compete with each other and what is the meaning and exact outcome of such competition.

Based on our cryo-EM analysis and FRAP data, it seems likely that the mammalian I-BAR domains do not form intermolecular contacts with adjacent domains or that these contacts are very transient. In contrast to the mammalian I-BARs, FRAP analysis revealed that in cells, the *C. elegans* I-BAR domain is likely to form stable lattices in membrane tubules. Additionally, the filopodia-like protrusions induced by *C. elegans* I-BAR domain appeared more rigid when compared with the mammalian I-BAR induced protrusions. Based on the data presented in publications I and II of this thesis, a schematic representation of the mechanism behind I-BAR domain-membrane interaction is presented (II Fig. 7). It is important to note, that these results were obtained by using constructs encoding for I-BAR domains and not the full-length proteins. Results presented in III and IV provide evidence that in the context of the full-length proteins, the C-terminal regions following the I-BAR domain may also play an important role in regulating the membrane interactions of the I-BAR proteins. Therefore, one should be careful not to make too far-reaching conclusions regarding the biological role of these proteins solely based on work with the isolated I-BAR domains.

Although the over-expression of all I-BAR domains tested so far results in dramatic formation filopodia in cells, only the role of endogenous IRSp53 in filopodia formation has been established beyond dispute (Lim et al., 2008; Krugmann et al., 2001; Disanza et al., 2006). Full-length IRSp53 localizes to the filopodia tips instead of the whole shaft, which is the case with IRSp53 I-BAR domain, indicating that the C-terminal domains (CRIB, SH3, WW, PDZ and WH2) play a role in controlling IRSp53 localization/biochemical activities. In filopodia, IRSp53 is activated by binding of Cdc42 to its CRIB domain. Via its SH3 domain, IRSp53 can interact with several regulators of the actin polymerization machinery including WAVE2 and N-WASP, whereas the C-terminal WH2 domain can potentially supply ATP-actin monomers to the site of growing actin filaments at filopodia tip (Scita et al., 2008; Ahmed et al., 2009). As IRSp53 forms a dimer through its I-BAR domain, it therefore harbors two WH2 domains in close vicinity. Although no structural information is available from the C-terminal region of IRSp53, it is tempting to speculate that when IRSp53 deforms membranes into tubular protrusions, the WH2 domains of adjacent IRSp53 molecules could possibly form actin nuclei, which would be sufficient to initiate the polymerization of actin filaments as in the case of actin nucleators that contain a tandem of WH2 domains (see chapter 1.5.1.1.). Alternatively, the WH2 domains can possibly act synergistically with I-BAR mediated membrane deformation by clustering actin filament barbed ends and/or by feeding monomers to the growing actin filaments thereby acting as actin filament elongation factors (Figure 9).

Interestingly, endogenous ABBA as well as the GFP-tagged full-length protein localize to distal part of lamellipodia in C6R cells. More importantly, siRNA mediated knockdown of ABBA alters lamellipodial dynamics (III). Similar results have also been obtained for IRSp53 (Suetsugu et al., 2006). This suggests that I-BAR proteins may control lamellipodia dynamics by either sensing curvature, which might be necessary to bring the right components together for efficient actin-based protrusion formation, or they might be able to generate proper membrane curvature to facilitate protrusion initiation/formation.
Figure 10. Current evidence suggests that during cell migration/extension, I-BAR proteins are utilized to control both lamellipodial and filopodial dynamics. Whether these activities occur parallel is currently unknown. One possibility is that the regulation between these two modes is I-BAR protein-concentration dependent. At low concentration, I-BAR proteins can possibly sense membrane curvature at the leading edge and bring together actin polymerization machinery to drive the formation of branched actin network promoting the formation of lamellipodial protrusions (Figure 10), whereas high I-BAR concentrations would lead to oligomerization and membrane tubulation activity that initiates filopodia formation (Figure 9). Selectivity between the different modes might also be controlled by intrinsic regulatory mechanisms (e.g. post-transcriptional modifications), by different binding partners or they might be cell-type specific responses.

In the case of membrane invaginations, the activity of N-WASP seems to be...
membrane curvature dependent (Takano et al., 2008). The link between IRSp53 and the actin polymerization machinery is well known, however this link is poorly established for other I-BAR members. MIM has been shown to interact with actin nucleation promoting factor cortactin through its polyproline region (Lin et al. 2005), implying that one way of defining the function of different I-BAR proteins might be obtained through differential selectivity towards distinct components of the actin polymerization machinery.

More recently, information about the role of I-BAR proteins in the regulation of intercellular adhesions has been obtained. Previously, it has been shown that IRSp53 functions at tight junctions (Massari et al., 2009). Our data presented in this thesis demonstrate that MIM is important for the maintenance of epithelial adhesive structures in kidneys. Loss of MIM results in gaps between the intercellular spaces of kidney epithelial cells and compromised function of these tubular cells. The intercellular adhesions are maintained and held together by specific adhesive structures such as tight and adherens junctions, desmosomes and the continuous polymerization of actin filaments against the lateral plasma membranes of adjacent cells (Jamora and Fuchs., 2002). Cell biological experiments with GFP-MIM confirmed that MIM localizes to intercellular adhesions in kidney epithelial cells and promotes actin filament assembly at these sites. Importantly, the I-BAR domain was necessary for the correct

Figure 10. Model how I-BAR proteins regulate plasma membrane dynamics. I-BAR domain localizes these proteins to their correct sites (e.g. by sensing membrane curvature) where they can potentiate actin dynamics by increasing \( \text{P}(4,5)\text{P}_2 \) concentration, which has a positive net influence on actin dynamics (Saarikangas et al., 2010). The regions between I-BAR and WH2 domains interact with NPFs, which can potentiate actin polymerization in a membrane curvature dependent manner (Suetsugu et al., 2009). Additionally, WH2 domains can increase the local ATP-monomer concentration.
localization of MIM and the WH2 domain enhanced the actin filament assembly at these sites. The Drosophila homolog of MIM/ABBA displayed a defect in collective cell migration of border cells. Collective cell migration takes place also during mammalian embryogenesis (reviewed in Friedl and Gilmour, 2009), however, no developmental defects were detected in MIM knockout mice. This might be due to functional redundancy in MIM knockout mice by ABBA or due to differences between the core functions of MIM/ABBA and the Drosophila homolog, which share very little homology at the amino acid sequence level outside the I-BAR and WH2 domains.

In addition to kidney, MIM is also expressed in other epithelial tissues such as liver epithelium (Mattila et al., 2003) and previous studies have found that expression of MIM is down-regulated in certain metastatic epithelial cancers (Lee et al., 2002; Ma et al., 2007). Our light-microscopic examination of epithelial tissues did not reveal any apparent changes in the epithelial organization of young MIM knockout mice, however, we have noticed an increased number of liver tumors in MIM knockout and heterozygous mice as compared to wild-type mice (unpublished data), indicating that epithelial cell-cell adhesions might also be defective outside the kidney.

Discussion

Figure 11. Hypothetical model for MIM in epithelial intercellular contacts. Integrity of intercellular contacts are maintained by cadherin-based adhesions and constant actin polymerization. The reparative mechanisms that regulate the re-establishment of cadherin adhesion might involve the formation of membrane protrusions through the action of MIM.
Discussion

epithelium. These findings fit nicely with our model for MIM, where we propose that MIM promotes intercellular adhesion integrity by optimizing and maintaining actin polymerization at these sites, possibly initiating reparative mechanisms to induce protrusion formation upon the break-up of intercellular cadherin adhesion, which would facilitate the formation of new adhesion (Figure 11). Thus, lack of MIM in epithelial cancer cells would be expected to result in weaker intercellular adhesions, which is one of the hallmarks of epithelial to mesenchymal transition (EMT) that describes a series of events that transform adherent epithelial cells into motile and invasive cells that can break through tissue barriers (reviewed in Kalluri and Weinberg, 2009).
FUTURE PERSPECTIVES

This thesis work set out to investigate the biology of the I-BAR proteins. The findings presented here contributed to the establishment of the I-BAR domains as novel membrane deforming modules. We have shown that the I-BAR domains bend membranes to the opposite direction compared to the canonical BAR domains and revealed interesting differences in the membrane deformation mechanisms between different I-BAR domains. In addition, we found that I-BAR proteins play important roles in various actin-dependent processes of specific cell types, such as radial glial cells and kidney epithelial cells. With each new finding, however, many more questions were raised and hence plenty of unresolved issues regarding the biology of the I-BAR proteins remain to be solved. For example, the dimerization status of I-BAR proteins in cells remains poorly characterized. In general, BAR domains are considered to function in a dimeric state, however, little is known about the possible differences in the activities of these proteins in their monomeric vs. dimeric states. The dissociation constants measured for the BAR and F-BAR domains have been in the range of 2.5-6 μM (Henne et al. 2007, Peter et al. 2004), indicating that the local cellular concentration of a given BAR protein must be relatively high in order for the dimerization to occur and presumably for the membrane deformation activity to take place. How are such high concentrations achieved and maintained at correct sub-cellular sites (e.g. at the leading edge of a migrating cell) remains a mystery. One possibility by which a local high concentration of BAR proteins could be achieved is through localized RNA translation (Holt and Bullock 2009). There is some evidence suggesting that at least the mRNA of ABBA is transported in radial glial cells.

The recent structure of full-length F-BAR protein pacsin/syndapin revealed a mechanism by which the membrane deformation activity of this protein is auto-inhibited (Rao et al. 2010). Similarly, structural data from the C-terminal part of I-BAR proteins could provide useful insights on how I-BAR proteins are self-regulated. Additionally, little if nothing is known about signals that contribute to the activation of these proteins. Therefore, it would be important to identify the interaction partners of I-BAR proteins, as well as map the possible post-translational modifications, which could contribute to the activation and/or inactivation of these proteins.

Probably the most fundamental and also the most challenging questions regarding the biology of I-BAR proteins relate to the actual function of the I-BAR module. It still remains an open question whether the function of the I-BAR domain is to actively generate membrane curvature or to sense and/or stabilize existing membrane curvature. It is interesting to note that both membrane curvature sensing and membrane deformation activities seem to be highly conserved mechanisms in evolution and play fundamental roles across species as these activities are also present in bacterial proteins (Ramamurthi et al. 2009; Tanaka et al. 2010). From the currently characterized cell biological roles of I-BAR proteins, one could make the assumption that both active membrane curvature generation as well as membrane curvature sensing can be important attributes in these proteins depending on the cellular process. For example, during cell migration, membrane curvature sensing by the I-BAR domain at the lamellipodium might be important for localizing the actin polymerization machinery to the desired localization at the right time, whereas...
Future perspectives
generation of membrane curvature could be important for nucleating the formation of new filopodia. In the case of persistent membrane nanotubules, I-BAR proteins might form a rigid scaffold that could support these long tubular plasma membrane extensions. The degree by which the biological functions of different I-BAR proteins and possibly some IF-BAR proteins overlap is currently unknown. Solving this question in a comprehensive manner would ideally require cross-breeding of different I-BAR/IF-BAR knock-out mice and thus represents a great challenge for the future research.

In conclusion, the I-BAR proteins represent a class of proteins that regulate the actin cytoskeleton and plasma membrane dynamics to induce cell protrusions in a novel way. Understanding the functions of these versatile proteins promises many new exiting chapters in cell biology and withholds keys for better understanding the basic principles that govern a number of different biological as well as pathological processes.
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[Signature]
REFERENCES


References


Itoh, T., J. Hasegawa, K. Tsujita, Y. Kanaho, and T. Takenawa. 2009. The tyrosine kinase

References
Fer is a downstream target of the PLD-PA pathway that regulates cell migration. *Sci. Signal.* 2:ra52.


References


References


References


Takenawa, T., and H. Miki. 2001. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin

References
Wang, Y., K. Zhou, X. Zeng, J. Lin, and X. Zhan. 2007b. Tyrosine phosphorylation of
missing in metastasis protein is implicated in platelet-derived growth factor-mediated cell shape changes. J. Biol. Chem. 282:7624-7631.


